The effect of iron and iron-binding proteins on murine and human lymphocytes

by

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ABBREVIATIONS

α-	anti-
ANAE	alpha-naphtyl acetate esterase
APC	antigen presenting cells
apoFt	apoferritin (free of iron)
apoLf	apolactoferrin (free of iron)
apoTf	apotransferrin (free of iron)
Arg	arginine
Asp	asparagine
ATP	adenosine triphosphate
BD	below detection
BSA	bovine serum albumin
C-	carboxy-
$[Ca^{2+}]i$	intracellular calcium concentration
CD	cluster of differentiation
cDNA	complimentary deoxyribonucleic acid
Ci	Curie
Con A	concanavalin A
cpm	counts per minute
CSA	colony-stimulating activity
CTL	cytotoxic T lymphocyte
Cys	cysteine
d	dalton
DFO	desferrioxamine
DG	diacyl glycerol
DNA	deoxyribonucleic acid
Fab	fragment obtained by papain hydrolysis of immunoglobulins (a pair of heavy and light chains)
FCS	fetal calf serum
FeNTA	ferric nitrilotriacetate
FePIH	ferric pyridoxal isonicotinoyl hydrazone
FeTf	ferric transferrin
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone
Ft	ferritin
GM-CSF	granulocyte/monocyte-colony stimulating factor

GTP	guanosine triphosphate
His	histidine
Hs	haemosiderin
HSA	human serum albumin
HTf	human transferrin
IEF	isoelectric focusing
IFN	interferon
Ig	immunoglobulin
IgG	immunoglobulin G
IL	interleukin
IL-2R	interleukin-2 receptor
IP	inositol phosphate
IRE	iron regulatory element
IRMA	immunoradiometric assay
IU	international unit
Kd	kilo dalton
Lf	lactoferrin
LfR	lactoferrin receptor
mA	milli-Ampere
Me	mercaptoethanol
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MLFt	mouse liver ferritin
MSFt	mouse spleen ferritin
MTf	mouse transferrin
mRNA	messenger ribonucleic acid
N-	amino-
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NK	natural killer
NRIg	normal rabbit immunoglobulins
NSIg	normal sheep immunoglobulins
NTA	nitrilotriacetate
P	probability of significance
p97	melano-transferrin
PBS	phosphate buffered saline
PDB	phorbol dibutyrate

PG	prostaglandin
PHA	phytohaemagglutinin
pI	isoelectric point
PIH	pyridoxal isonicotinoyl hydrazone
PIP-PDE	polyphosphoinositide phosphodiesterase
PKC	protein kinase C
PMA	phorbol myristate acetate
PMSF	phenylmethyl sulphonyl fluoride
Rα-MLFt	rabbit antimouse liver ferritin
Rα-MSFt	rabbit antimouse spleen ferritin
rpm	revolutions per minute
RPMI	Roswell Park Memorial institute (medium number 1640)
S-	Sepharose 4B coupled to-
Sα-HTf	sheep antihuman transferrin
sat.	saturation
SD	standard deviation from the mean
SDS	sodium dodecyl sulphate
T X-100	Triton X 100
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tf	transferrin
TfR	transferrin receptor
Ti	antigen receptor
Tyr	tyrosine
UTR	untranslated region
v/v	volume per volume
w/o	without
w/v	weight per volume
Å	Ångstrom

SUMMARY

It is well documented that iron deficiency affects the function of lymphocytes because this essential element is required by these cells. However, the way that lymphocytes respond to high levels of iron is not fully understood. Reports of an increased incidence of infection and neoplasia among iron-overloaded patients might indicate that excess iron causes depression of specific immune responses in these patients. The broad objective of these study was therefore to investigate how lymphocytes react to different degrees of extracellular iron availability.

Iron uptake by proliferating mouse lymphocytes from transferrin (Tf) saturated to different degrees with iron showed a gradual increase at saturations below complete saturation of the protein. The uptake rose sharply when non-Tf bound iron was present in the medium and the ratio of iron uptake to iron available increased. The proliferative capacity of these cells assayed alongside iron uptake was low at low Tf saturations and an increased rate of transformation was associated with increased percentage of saturation of Tf with iron. When saturation exceeded the binding capacity of the protein, proliferation decreased and at high levels of iron it was reduced below control level. Ferric nitrilotriacetate (FeNTA) was found to donate very large amounts of iron to cells compared to Tf but did not promote proliferation and when present in high amounts caused inhibition. In contrast ferric pyridoxal isonicotinoyl hydrazone (FePIH) which donated iron to cells at a slightly higher rate than Tf was found to support proliferation as efficiently as Tf.

A study has been made of intracellular events in the iron

metabolism of proliferating mouse lymphocytes to clarify the relationship between iron uptake and intracellular iron metabolism in these cells cultured with different iron carriers and to relate this to their ability to promote proliferation. This involved iron chelation, immunoprecipitation and ultrafiltration. In cells cultured with FeNTA, iron was found predominantly in an insoluble non-Ferritin (Ft) macromolecular form, while in the cells cultured with FeTf or FePIH the largest proportion of iron was found in the intermediate molecular weight fraction, which probably represents iron being used to form enzymes. The cells showed no marked increase in synthesis of Ft irrespective of the form of iron present. Mouse lymph node cells were also found to contain endogenous Tf. Synthesis of Tf was found in *in vivo*-stimulated lymphocytes, and macrophages were found to be the most active cells in synthesising this protein.

Comparable studies of the effect of different iron carriers on cellular proliferation were performed with human lymphocytes and a related cell line, the T-lymphoblastoid CCRF-CEM line. Generally human lymphocytes gave similar results to mouse cells except with FePIH which was found to be less effective than with mouse cells. In addition, the presence of non-Tf bound iron in the form of FeNTA, but not FePIH, caused a decrease in CD4/CD8 ratio, due mainly to depression of the proportion of $CD4^+$ cells. However unlike normal cells CCRF-CEM cells did not show any difference in their proliferative activity at different saturations of Tf and were able to achieve good proliferation when high levels of non-Tf bound iron in the form of FeNTA or FePIH was present in the culture medium. These cells were also found to have the ability to make their own Tf.

Unlike Tf, Lf, the other member of the Tf family did not have any

effect on proliferation of stimulated human lymphocytes whether added in the (apo) or loaded from. However, in the presence of excess non-Tf bound iron, apoLf increased the ability of these cells to proliferate.

Therefore the main conclusion of this study is that the presence of concentrations of iron above the level that saturates all transferrin present in the medium inhibits proliferation of lymphocytes, and preferentially affects the helper subsets. It seems likely that some mechanism for uptake of unbound iron into these cells may exist resulting in toxic consequences.

CHAPTER ONE

PART ONE

LITERATURE REVIEW OF IRON METABOLISM

1.1.1 Role of iron in living organisms

Iron, is the second most common metal in the earth's crust and an invariable essential constituent of all living organisms with possibly the exception of the lactobacilli (Archibald, 1983).

Like other transition metals, iron is an important biological catalyst. Because it possesses unfilled *d* atomic orbitals, iron has oxidation states varying from -2 to +6, but normally it exists in the equilibrium between the +2 and +3 oxidation states, an equilibrium which is very sensitive to both ligation and pH (Hider, 1984). These changes in oxidation states involving one electron allow iron to coordinate electron donors and play a crucial role in many biological processes requiring oxidation and reduction.

The thermodynamically stable state of iron is Fe^{3+}, but at concentrations greater than 10^{-18} M, which is the equilibrium concentration of the ion at neutral pH, uncomplexed Fe^{3+} ions are very readily hydrolysed and polymerised to form essentially insoluble rust-like, ferric hydroxides and oxyhydroxide polymers (May and Williams, 1980).

The evolution of organisms shows a long dependence on iron, and nature has developed rather sophisticated iron chelating and transport systems to utilize the mineral as well as storage systems, though many organisms are auxotrophic for Fe^{3+}. Microorganisms tend to utilize small non-proteinaceous molecules to ensure an adequate supply of the metal. Bacteria have developed a very efficient apparatus for gaining access to

this element, involving a group of low molecular weight high affinity chelating agents named siderophores for sequestering and transporting iron from the surrounding environment (Neilands, 1981). These are virtually ferric ion-specific ligands, and usually catechols or hydroxamates. Plants also synthesise and secrete substances that augment iron absorption from the external environment (Finch and Huebers, 1982).

In mammals, the iron binding functions are met by more complicated molecules that form reversible complexes with the metal. These include a group of very similar iron-transport proteins called transferrins (Tfs) and two other proteins, ferritin (Ft) and its lysosomal degradation product haemosiderin (Hs), which fulfill the role of maintaining iron in an available form, but which are essentially iron storage proteins. Beside the group of iron transport and storage proteins, there are two other groups of iron-containing proteins i.e. those which complex oxygen and transport it throughout the body, and iron containing enzymes. There are data showing the existence of siderophore-like low molecular weight iron binding ligands with high binding specificity for Fe^{3+} at neutral pH in mammalian cells, which are preferentially produced under conditions of iron deprivation such as iron deficiency, anaemia, or in pregnant women (Apte and Brown, 1969). Fernandez-Pol (1978) has identified and partially purified a highly specific iron-binding ligand from a mutant of SV-40 transformed mouse cells adapted to grow in picolinic acid, which consists of a peptide of approximately 1.6 Kd.

In all biological systems, iron is always found bound to

complexing agents. This is not only to permit the use of the element in a wide variety of metabolic processes, but also to prevent its potentially deleterious effect which is expressed in the form of free radical formation. It is well known that iron enhances the toxicity of oxygen free radicals (McCord and Day, 1978; Halliwell, 1978). These free radicals are highly reactive and could be very harmful to the host.

There is no doubt that almost all living cells, prokaryotes, eukaryotes, aerobic, anaerobic, photosynthetic, and nitrogen-fixing, cannot survive without iron. Iron plays a crucial role in modulating oxygen supply and transport. Most of the proteins responsible for oxygen binding (haemoglobin and myoglobin) use iron to enable them to bind oxygen reversibly, the few exceptions being found in certain molluscs and arthropods, which use copper in haemocyanin as an oxygen carrier.

Iron enables other haem-containing proteins to participate in several essentially biochemical reactions with molecular oxygen, such as transfer of electrons in cellular respiration, the reduction of peroxides, oxygenation and hydrogenation of organic substances, as well as the transfer of electrons between dehydrogenases and various electron acceptors (Griffiths, 1987).

Iron is also present in a number of enzymes known as iron-sulphur proteins, and through them participates in a number of redox reactions involved in the biosynthesis of steroid hormones, oxidative phosphorylation reactions, and in non-redox reactions involved in the Krebs cycle and purine synthesis (Griffiths, 1987).

The process of DNA replication depends on iron through a non-haem iron-containing enzyme, ribonucleotide reductase which catalyses the conversion of ribonucleotides to deoxyribonucleotides, an essential step in DNA synthesis (Reichard and Ehrenberg, 1983). Iron seems not to participate as an electron carrier in the process of DNA synthesis, but helps to generate and stabilize the organic radical intermediate during catalysis (Atkin *et al.*, 1973), which is necessary for the activity of the enzyme.

The total body iron content of a man of 70 Kg weight is about 4.2 g. Haemoglobin normally accounts for 74.3%, myoglobin 3.3%, haptoglobin and haemoglobin 0.2%, Tf iron accounts for only 0.07%, catalase 0.11%, cytochrome C 0.08%, and the remaining 16.4% is distributed between the proteins of storage Ft and Hs (Beinert, 1973). This distribution of the body iron rapidly changes in pathological disturbances such as iron deficiency and iron overload.

1.1.2 The transferrins: general properties, synthesis, and function

The transferrin protein family consists of a group of evolutionarily and therefore structurally-related metalloproteins, which are found only in the phylum of chordata. These are transferrin (Tf), lactoferrin (Lf), ovotransferrin (ovo-Tf), and another one recently discovered; melanotransferrin (melano-Tf). They are present and widely distributed in numerous vertebrate biological fluids (Feeney and Komatsu, 1966; Aisen and Listowsky, 1980). They are monomeric glycoproteins which

can reversibly bind two atoms of Fe^{3+} per molecule of protein, so they may exist in the apo-, mono-, and diferric forms.

Plasma transferrin carries iron and transports it from the intestinal mucosa (site of absorption), the reticuloendothelial system (site of erythrocyte catabolism), and the liver parenchymal cells (site of storage) to supply most body tissues with iron. The function of the other related protein lactoferrin remains unclear, and there is little evidence of an iron-transport role, but it may, like Tf, contribute to antimicrobial defences. Ovotransferrin was the first of the group to be found in 1889 (cited by Weinberg, 1978) in eggwhite from which its name was derived.

1.1.2.1 Transferrin

The major circulating iron binding protein was isolated and characterised in 1946 by Schade and Caroline. The following year, the protein was named transferrin and its physiological importance in the transport of iron in the circulation and also into the cell was confirmed (Laurell and Ingelman, 1947).

The total amount of Tf in man has been estimated to be about 240 mg/Kg body weight. The normal blood concentration of Tf is about 30 μM, at an iron saturation of about 30%, with half life of about 8-10 days. The concentration of Tf in the serum was found to increase progressively throughout fetal life from about 0.4 mg/ml at 10-15 weeks gestation to levels greater than that of the mother near term (Morgan, 1964; Baker and Morgan, 1969; Wong and Morgan, 1973). Tf is found throughout the extracellular fluids, being equally divided between the intra- and extravascular compartment. It has also been identified in

lymph (von Ehrenstein, 1956), pleural, ascitic, oedema (Bogdanikowa and Grabowski, 1972), and cerebrospinal fluid (Parker *et al.*, 1963). Tf is present in the milk of certain animals such as the rat (Morgan, 1981), rabbit (Suard *et al.*, 1983), and mouse (Lee *et al.*, 1987), cow (Sanchez *et al.*, 1988). It has been reported that in milk of some of these animals Tf predominates, rather than Lf (see below).

Tf is synthesised mainly in the liver (Morgan and Peters, 1971; Morton and Tavill, 1977; McKnight *et al.*, 1980). Tf cDNA from a human liver library has been identified, characterised, and the Tf gene mapped on human chromosome 3 (Yang *et al.*, 1984). In the fetus Tf synthesis may occur in the yolk sac (Yeoh and Morgan, 1974) and the lung (Gitlin and Biasucci, 1969) as well as in the liver. The newly synthesised Tf in the hepatocyte contains an additional leader sequence of some 19-20 amino acids, which is split by proteolysis prior to secretion of the protein (Thibodeau *et al.*, 1978; Jeltsch and Chambon, 1982). It seems that this latter step is crucial in the synthetic process, since inhibition of proteolysis prevents secretion (Schreiber *et al.*, 1979). The final step in Tf synthesis is glycosylation, which takes place in the rough endoplasmic reticulum and the Golgi system while *en route* to be secreted before it enters circulation. Omission of this step seems to have little effect on the secretion process (Schreiber *et al.*, 1979). The rate of Tf synthesis by human liver was determined to be 10.5% of the plasma pool per day (Wochner *et al.*, 1968; Kernof and Baker, 1980).

Other nonhepatic cell types have the ability to make Tf. Synthesis of the protein in extrahepatic sites probably contributes to the requirements of specific tissues, which are separated from plasma by

blood barriers that may prevent adequate Tf from being supplied by the circulation, in inflammation, and in iron overload (Bowman *et al.*, 1988).

Ovary cells (Thorbecke *et al.*, 1973), as well as testis Sertoli cells (Skinner and Griswold, 1980; 1982; Skinner *et al.*, 1984) which are responsible for secretion of most of the fluid components of the seminiferous tubules, synthesise a Tf-like protein *in vitro* differing from serum Tf only in the glycan composition. However it has been suggested that *in vivo*, Sertoli cells are not actively engaged in the synthesis of testicular Tf and that upon *in vitro* culturing Sertoli cells activate the Tf gene (Lee *et al.*, 1986). In chicken, Tf is synthesised by the oviduct and its transcription is induced by oestrogens (Lee *et al.*, 1978). Fibroblasts make Tf (Stecher and Thorbecke, 1967b), as also do macrophages (Stecher and Thorbecke, 1967a; Haurani *et al.*, 1973), and the muscle (Levin *et al.*, 1984). Tf mRNA has been found at low levels in rat placenta, spleen, kidney, muscle, and heart (Aldred *et al.*, 1987). It has also been found in newborn, but not adult intestine (Levin *et al.*, 1984). Tf has been described as a fetal growth factor in embryonic induction (Ekblom *et al.*, 1983). It has also been identified as a major protein synthesised and secreted by the mammary epithelium of certain animals, which is identical to the corresponding plasma homologue except for a lower content of sialic acid (Baker *et al.*, 1968; Lee *et al.*, 1987). It has been reported that in late lactation rat milk contains as much as 4-5 mg/ml Tf. The regulation of its synthesis and secretion is distinct from that of other milk proteins (Lee *et al.*, 1987), and exercised at the mRNA level. The central nervous system has been found to make Tf (Bloch *et al.*, 1985; Dickson *et al.*, 1985). It has

been found not only in oligoendrocytes and glial cells, but also in neurons and endothelial cells (Møllgård *et al.*, 1987), and has been described as acting as a neurotrophic factor (Beach *et al.*, 1983). Nevertheless, the amount of Tf synthesised by extrahepatic sites is far less than that produced by the liver. Idzerda *et al* (1986) measured Tf mRNA levels in various tissues of normal rats and found 6500 molecules/cell in liver, compared with 114 in testis, 83 in brain, 11 in spleen , and 5 in kidney.

As far as lymphocytes are concerned, early studies have shown that human lymph nodes (Prunier *et al.*, 1964) and lymphocytic cell lines (Stecher and Thorbecke, 1967b) make Tf. It has been shown that there is an association of radiolabelled immunoreactive Tf with cell extracts of peripheral mononuclear cells at periods of both pre- and post-activation, although the molecular weight was not determined (Soltys and Brody, 1970). More precisely the release of Tf has been demonstrated to be from $OKT8^+$ subpopulations of T-lymphocytes (Broxmeyer *et al.*, 1983). In contrast to Broxmeyer's findings, another group has demonstrated the transcription and synthesis of Tf by T-lymphocytes and localised Tf mRNA specifically in $T4^+$ helper-inducer human lymphocyte subsets, whereas no Tf mRNA was detected in $T8^+$ or in B-cells (Lum *et al.*, 1985; 1986). In peripheral blood mononuclear cells, approximately 2-5% were seen having silver grains denoting Tf mRNA hybridization by using *in situ* hybridization, and after T-cells were isolated by rosette formation with sheep red blood cells, 10-20% of the T-cells transcribed Tf mRNA (Bowman and Yang, 1987). Cloned Tf-independent lymphoma cell lines have been isolated and reported also to

produce a Tf-like activity (Morrone *et al.*, 1988).

Childhood, pregnancy, iron deficiency anaemia, oestrogen, and administration of some steroid hormones (Bowman *et al.*, 1988) are factors which increase plasma Tf levels. In the liver, oestrogen stimulates Tf synthesis by increasing mRNA transcription (McKnight *et al.*, 1980). Tf synthesis by the testis appears to be hormone control-independent (Perez-Infante *et al.*, 1986). However other workers have shown that there is a regulation by various hormones, such as FSH, testosterone, and insulin (Skinner and Griswold, 1982), and that this modulation of the Tf expression is exerted on a transcriptional level (Huggenvik *et al.*, 1987). Iron deficiency is an other factor that increases plasma Tf concentration. An iron deficient diet induces an increase of 2-4-fold in serum Tf as well as in Tf mRNA synthesis in the liver (McKnight *et al.*, 1980). In isolated liver nuclei of rats raised on low iron diet, a 2.4-fold increase in Tf transcription activity was found but without physiologic effect since serum Tf iron binding capacity was unchanged (Idzerda *et al.*, 1986). However, the Tf mRNA content of other Tf synthesising tissues such as brain, testis, spleen, and kidney remained unchanged (Idzerda *et al.*, 1986). It has been reported that iron overload had no detectable effect on Tf gene expression (Tuil *et al.*, 1985). However, recently Lescoat *et al* (1989) have demonstrated that iron overload decreases Tf secretion and postulated that this is exerted through a translational control of the protein.

Very little is known about the precise mechanism or site of catabolism under normal conditions. A proportion (10%) of breakdown has been found to be hepatic as suggested by isolated perfused liver

(Hoffenberg *et al.*, 1970). The catabolic rate of plasma Tf is 6-14%/day of plasma pool (Morgan, 1983) which corresponds closely to determinations of the rate of Tf synthesis.

The main function of Tf is to transport iron from sites of storage, absorption, and erythroid catabolism to specialized iron requiring cells, mainly erythroid precursors for haemoglobin synthesis, and specialized iron storage cells (hepatocytes), which incorporate iron into ferritin. The role of Tf in cellular uptake is described in detail in section 1.1.5.2.

Tf also donates iron to actively proliferating cells from different origins. Activated lymphocytes are an example of these cells. Although the amount of iron taken up by those cells is relatively small, this process plays an important role in cell transformation and division. One of the most crucial roles of iron as far as those cells are concerned is its association with ribonucleotide reductase (Brown *et al.*, 1969). However, others have suggested that the Tf growth factor effect is exerted through acting as an electron acceptor for a transmembrane redox system involving NAD (Crane *et al.*, 1985; Navas *et al.*, 1986). Sun *et al* (1987) have reported that the NADH reductase functions in association with the TfR, where Tf attains its effects through modulation of transmembrane potential differences. The role of Tf in lymphocyte proliferation is described in detail in section 1.2.3.

1.1.2.2 Lactoferrin

Lf, which appears to be restricted to mammals, is another member of the Tf family. It is also called lactotransferrin by analogy to its plasma homologue. Lf was first found in its most abundant source, milk

(Montreuil *et al.*, 1960). Subsequently, Lf has been found in most mammalian secretions, including bronchial, nasal, salivary, lachrymal, genital, and gastro intestinal secretions, as well as in sweat, tears, and pancreatic juice (Figarella and Sarles, 1975; Aisen and Listowsky 1980), so it is constantly bathing mucous membranes which are vulnerable to infection. Lf is also found in neutrophils (Baggiolini *et al.*, 1970), more precisely in the specific (secondary) granules (Cramer *et al.*, 1985; Esaguy *et al.*, 1989). During degranulation of neutrophils, Lf is released not only to the phagocyte vacuole but also to extracellular medium. In human plasma, it is present in minute amounts (Bennett and Mohla, 1976). Whether Lf plays any significant role in the transport of iron is not known. If it does, the pathway is probably from plasma to reticuloendothelial cells (Morgan, 1980). Lf is a major constituent of milk and human colostrum contains high concentrations, up to 15 mg/ml. Lf in human milk is less than 5% saturated with iron (Lonnerdal, 1985). Lf was also found in amniotic fluid (Niemelä *et al.*, 1989). Plasma concentration of Lf is only about 1 μg/ml (Bennett and Mohla, 1976). Sykes *et al.* (1982) found that the serum Lf level is higher in pregnancy than in normal adult women. Lf escapes digestion and any detrimental effect of the low pH, as judged by the large amount of the so-called coprolactoferrin daily excretion in faeces (5-35 mg) (Spik and Montreuil, 1966; Spik *et al.*, 1982).

Like Tf, genes coding for human Lf are located on the long arm of chromosome 3 (Teng *et al.*, 1987), while mouse Lf genes lie on band q21-25 of chromosome 9 (Yang *et al.*, 1984). Human Lf shows 59 and 49% sequence homology with human Tf and hen ovo-Tf respectively (Metz-Boutigue, 1984). On the other hand, Lf shows marked

differences from Tf in antigenic determinants, tryptic peptide patterns, isoelectric point, the disposition and composition of the carbohydrate moieties (see section 1.1.3), and finally Lf has a much higher affinity for Fe^{3+} than that of Tf (Aisen and Leibman, 1972).

Lf is found totally or partially complexed to 80% of lysozyme present in milk probably with a ratio of 2 molecules of lysozyme to one of Lf and it complexes also with some sialyted glycopeptides of unknown role (Montreuil *et al.*, 1985). It is also found to be associated with secretory IgA (Watanabe *et al.*, 1984) by ionic interaction, and to an antitryptic factor (Montreuil *et al.*, 1985). It has also been observed that human Lf binds to murine macrophages and the presence of specific receptors has been reported (van Snick and Masson, 1976; Birgens *et al.*, 1983). The binding of Lf to adenocarcinoma cells (Amouric *et al.*, 1984), to lymphocytic cell lines (Hashizume *et al.*, 1983), and to human T-lymphocytes (Mazurier et al., 1989) has also been reported.

The major function of Lf is thought to involve bacteriostatic activity (Oram and Reiter, 1968) by a mechanism of iron deprivation, and perhaps in concert with other compounds such as sIgA (Oram and Reiter, 1968; Bullen *et al.*, 1972; Rogers and Synge, 1978), or lysozyme (Montreuil *et al.*, 1985), which represent a powerful system for defence of mucosae. It has been suggested that in the small intestine Lf would facilitate iron absorption by donating iron to specific receptors at the brush border membrane (Cox *et al.*, 1979; Mazurier *et al.*, 1985; Lonnerdal, 1985), although others propose an inhibitory effect (Brock, 1980). Nevertheless, a specific LfR was identified in the mouse small intestinal brush-border membrane (Hu *et al.*, 1988). In neutrophils Lf

may function as a regulator of granulopoisis (Broxmeyer, 1989; Fletcher, 1989). It has been reported that Lf regulates the adhesion of neutrophils during the inflammatory response, by promoting aggregation and adherence of those cells to endothelial cells (Oseas *et al.*, 1981). Lf also enhances adherent natural killer cell cytotoxicity, while inhibiting antibody dependent cellular cytotoxicity (Nishiya and Horwitz, 1982). Lf also suppresses antibody production (Duncan and McArthur, 1981) as well as inhibiting colony stimulating factor (CSF) production (Broxmeyer *et al.*, 1978) All these data suggest that Lf may play a role in regulation of the immune response. It has also been reported that Lf may enhance cell proliferation of a human colon adenocarcinoma cell line in the presence of low concentrations of iron (Amouric *et al.*, 1984), and stimulates proliferation of rat crypt cells (Nichols *et al.*, 1987). Recently Mazurier *et al.*, (1989) have reported that PHA-stimulated human lymphocytes express specific surface receptors for Lf, and that the addition of Lf increased the proliferative activity of these cells in a similar way to Tf. However, others claim that it suppresses T-cell proliferation induced by mixed lymphocyte culture (MLC) or (PHA) (Slater and Fletcher, 1987), and that the mechanisms of suppression involve the chelating property of Lf (Richie *et al.*, 1987).

1.1.3 Structure and iron binding properties of the transferrins

Transferrins evolved from a smaller ancestor some 200-500 million years ago, which was probably a polypeptide chain one half the size of the contemporary vertebrate protein (Williams, 1982). This notion is

supported by the homology found in the amino acid sequence of the carboxyl halves of vertebrate Tfs (Metz-Boutigue *et al.*, 1984). Following an intragenic duplication that produced the amplified Tf gene, the Tf molecule gained an additional half and another iron binding site, and gave one chain with potential homology between the two halves (MacGillivray *et al.*, 1983; Park *et al.*, 1985). Amino acid sequencing and fragmentation, and X-ray diffraction studies, have concluded that Tfs are folded to form two more or less independent globular lobes, each containing one of the iron-binding sites at equivalent locations (MacGillivray *et al.*, 1983; Anderson *et al.*, 1987; 1989; Bailey *et al.*, 1988). They are connected by a short α-helix consisting of 7 residues in Tf and 9 in ovoTf. Each lobe contains two domains, with the iron binding site at the interface in highly hydrophilic environment.

Full length cDNAs have been cloned for human and rat serum Tf (Yang *et al.*, 1984; Uzan *et al.*, 1984; Levin *et al.*, 1984), chicken ovo-Tf (Jeltsch and Chambon, 1982), and melano-Tf (Rose *et al.*, 1986). Human Tf cDNA has an open frame encoding a protein of 679 amino acids plus the leading sequence of 19 amino acids which probably constitutes a signal for protein secretion since it is not found in the mature protein (Yang *et al.*, 1984; Uzan et al., 1984). A homology of 35-40% was found between amino acids 1-336 and 337-679 in human Tf. The presence of two lobes in the Tf molecule with this high degree of homology has been demonstrated by several techniques (Williams, 1975; Brock and Arzabe, 1976; Lineback-Zins and Brew, 1980; MacGillivray *et al.*, 1983; Anderson *et al.*, 1987). The corresponding lobes in Lf comprise residues 1-332 and 342-686. Melano-Tf has in addition an extra

25 amino acid residues at the C-terminus. This peptide has highly hydrophobic characteristics and is thought to act as a membrane anchor. Greater homology has been found (46%) between the two domains of melano-Tf (Rose *et al.*, 1986).

The N-terminal lobe of all the Tfs has been found to contain 6 disulphide bridges, whereas the C-terminal lobe of Tf contains 13 against 9 for Lf and 10 for ovo-Tf (Montreuil *et al.*, 1985). Beside this difference in the number, there are differences in the localization on each lobe i.e. whether they are local or widely spaced. These bridges serve to stabilize the conformation of the molecule and make its structure compact.

Tfs contain approximately 6% carbohydrate. The attachment sites are widely distributed over the molecule surface (Baker *et al.*, 1987). Human Tf and Lf contain two glycans of the N-acetyllactosaminic type (Spik 1982; Spik *et al.*, 1985), while ovo-Tf contains only one glycan chain (Dorland *et al.*, 1979; Montreuil *et al.*, 1985). The two glycosylated sites of Tf are located in the C-terminal half at residues 413 and 611 (MacGillivray *et al.*, 1983), while in Lf each site is present in each lobe of the protein (residues 137 and 478) (Baker *et al.*, 1987). The only glycan site of ovo-Tf is located in the C-terminal part (residue 473) (Williams *et al.*, 1982; Metz Boutigue *et al.*, 1984). Little is known about the role of these glycan structures in the physiological function of the protein.

The summation of the polypeptide chain of 679 amino acid residues and the two N-linked complex type glycan chains results in a calculated molecular weight of 79.570 D. Human serum-Tf has an isoelectric point

(pI) ranging between 5.6-5.9 (Hovanessian and Awdeh, 1976). Lf has a much higher pI ranging from 8.2-9.6 (Kinkade *et al.*, 1976). This is due to the presence only on Lf of a large number of basic residues at the surface of the molecule (Anderson *et al.*, 1989). OvoTf has a pI similar to that of serum Tf (Bain and Deutsch, 1948).

Tfs have two specific sites for iron. Therefore, Tfs may exist in the apo-, monoferric (with either the N- or C-terminal binding site occupied), and diferric form. Earlier studies have shown that the sites are not equally populated. It has been shown that at physiological pH, there is a preferential occupation of the N-terminal domain by iron (Leibman and Aisen, 1979). Zak and Aisen (1986) have found a considerable range in the ratio of occupancies of N-terminal and C-terminal sites between 21 normal human serum specimens with the N-terminal site predominantly occupied in most subjects. However, van Eijk and van Noort (1986) demonstrated that there is a shift of iron from the N- to the C-terminal binding site in Tf of stored human serum, but not in comparable preparations of purified Tf in PBS. On the other hand, it has been reported that the two sites are equally populated in the circulation (Williams and Moreton, 1980) by using urea gel electrophoresis techniques. Another report based on isoelectric focusing (IEF), has suggested that iron is randomly distributed, implying that the two sites are equally occupied whatever the level of saturation (Huebers *et al.*, 1984). The distribution of iron donated to apo-Tf is dependent on the nature of the presenting chelate. When iron was added to rabbit Tf as a complex with nitrilotriacetic acid (FeNTA), the C-terminal binding site was occupied, whereas iron added as ferric citrate occupied the N-

terminal site (Heaphy and Williams, 1982).

Apo-Tfs are colourless, but develop a red brown colour with maximum absorbance at 465-470 nm as the iron is bound (Schlabach and Bates, 1975). Apart from iron, other cations can bind to Tfs, including copper, vanadium, chromium, aluminum, cobalt, gallium, manganese, magnesium, plutonium, indium, zinc, terbium, europium, platinum, americium, and curium (reviewed by Huebers and Finch, 1987). The binding of iron to apo-Tf leads to conformational changes in the molecule, which becomes less susceptible to proteolytic degradation and denaturation (Makey and Seal, 1976; Hovanessian and Awdeh, 1976; Esparza and Brock, 1980). It has also been noticed that some antigenic sites of apo-Tf become hidden when iron is bound to the molecule (Tengerdy *et al.*, 1966).

The affinity of iron binding by Tf is maximal under physiological conditions (Morgan, 1981), the association constant for human Tf being about 10^{20} M^{-1} (Aisen *et al.*, 1978), and that for Lf some 26 times greater (Aisen and Leibman, 1972). However, the affinity decreases as the pH is reduced. It is frequently stated that iron starts to dissociate from the molecule of Tf at pH 5-6, and is completely released at pH 4.5 (Morgan, 1981). Lactoferrin does not release iron unless the pH is reduced to about 2 (Masson and Heremans, 1968). Iron is also lost at high pH, about 9-10 (Zapolski and Princiotto, 1980). However in the presence of competing chelators this might vary considerably. The iron binding sites of Tfs may differ in their binding affinities. At pH 6.7, the affinity of the N-terminal site of the human Tf for iron is less than 1/20 that of the C-terminal site (Evans and Williams, 1978), but at pH 7.4 the

affinity differs by a factor of only 5 or 6.

The requirement for synergistic binding of an anion for the binding of each iron to the molecule is now well established. The recent crystallographic structure analysis of Anderson *et al* (1989) favoured a carbonate to be the most likely anion involved in the binding of iron.

The high resolution of the Lf molecule (3.2 Å) of Anderson *et al* (1987) and the more recent one (2.8 Å) (Anderson *et al.*, 1989), and rabbit serum Tf (3.3 Å) of Bailey *et al* (1988) have identified the amino acid residues involved in the binding of iron and the anion. Each iron is coordinated by four protein ligands, which is in accordance with the earlier finding of Chasteen (1983). According to Bailey *et al* (1988) these consist of phenolate oxygens of two tyrosines 93 and 191 (Tyr 447 and 540 in C-lobe), which is in line with what Pecoraro *et al.* (1981) have reported; the imidazole nitrogen of one histidine 252 (His 609 in C-lobe), which was also reported earlier (Krysteva *et al.*, 1975; Rogers *et al.*, 1977; Zweier and Aisen, 1977) and the carboxylate oxygen of asparatate 61 (Asp 407 in C-lobe) plus a probable CO_3^{2-} or HCO_3^- anion bound to iron and to an adjacent arginine side chain 121 (Arg 477) which is highly conserved among Tfs. This is in contrast with earlier work of Chasteen (1983) who reported that two histidines play a role in the iron binding site. The remaining coordination site of the iron atom is linked to a water molecule.

1.1.4 Iron-storage proteins

1.1.4.1 Ferritin

As was mentioned above (see section 1.1.1), ionic iron is capable of mediating reduction of oxygen, giving rise to unstable intermediates. These potentially noxious compounds are known as free radicals which are highly reactive and could be very harmful. Therefore, an elaborate system of segregation and storage of iron is necessary to prevent iron toxicity and allow reutilization of stored iron when needed. This essential task is accomplished by the storage iron protein ferritin. Haemosiderin, its lysosomal degradation product, is also thought to play this role (see next section).

Ft is an iron storage protein used to maintain iron in an available non-toxic form within the cell. It is found in most cell types of vertebrates, in higher plants, fungi, and bacteria (Theil, 1987). The iron-free protein apo-Ft, which has a molecular weight of 450 Kd (Harrison *et al.*, 1967) is a roughly spherical coat with an outer diameter of about 125 Å, surrounding a core of about 70-80 Å across which houses iron atoms as microcrystalline polymers of ferric hydroxyphosphate (Ford *et al.*, 1984). The molecule of Ft consists of 24 subunits arranged in a form that leaves 6 channels 3-4 Å wide (Harrison *et al.*, 1980), through which iron can pass into and out of the molecule. These channels play a crucial role in the the iron binding process which is helped by side chains of amino acid residues at the wall of these channels. The central cavity can store up to 4500 iron atoms together with variable amounts of phosphate (Mann *et al.*, 1986).

The Ft molecule is made of different sorts of subunits. The isoferritins are hybrid molecules composed of varying proportions of these different subunits. Three subunits have been described (reviewed by Arosio, 1989). The light (L) subunit, is made of 174 amino acids, has a molecular weight of 19 Kd and a high pI, and is predominant in iron loaded tissues eg. the liver and spleen. The heavy (H) subunit, is more acidic than the L one, is made up of 182 amino acids and has a molecular weight of 21 Kd and a lower pI. It is predominant in iron poor tissues eg. heart, lymphoid cells, and malignant cells. Finally the G subunit (for glycosylated), with a molecular weight of 24 Kd, has been isolated from human serum (Cragg *et al.,* 1981), but small amounts may also be present in tissues. L-chains from horse, rat, and human, share about 85% of sequence homology, while H- and L-chains differ by about 45% (Costanzo *et al.*, 1984; Leibold *et al.*, 1984; Boyd *et al.*, 1985).

Ft is present in serum and can be used as a clinical indication of iron status (Worwood, 1986). It is also found in small amounts in milk (Arosio *et al.*, 1984). Adult human heart, kidney, pancreas, and placental Fts as well as Ft derived from neoplasms, contain more acidic iso-Fts, which are rich in H-chains, than liver and spleen Fts, which are more basic and rich in L-chains (Drysdale, 1970).

In humans, the H- and L-chains of Ft are derived from multigene families. Southern blot analyses and chromosomal *in situ* hybridization, indicate that there are about 15 H-chain Ft sequences and 5 L-chain Ft sequences in human DNA (Cragg *et al.,* 1985; Jain *et al.,* 1985). These are found on at least 7 different chromosomes for the Ft H genes

and on 3 chromosomes for the Ft L genes (McGill *et al.*, 1987).

Both the channels and the core fulfill the role of binding iron to the molecule. When Fe^{2+} atoms are added to apo-Ft they migrate to the interior by passing through the channels, probably aided by amino acid side-chains at the surface of these channels. Once in, Fe^{2+} is oxidized to Fe^{3+} and polymerises to form iron-core nucleation clusters (Bakker and Boyer, 1986). The loss of iron from the core via the channels requires reducing agents to form ferrous iron such as dithionite or flavines (Funk *et al.*, 1985) or free radicals (Thomas and Aust, 1986). Subsequently, the ferrous iron leaves the Ft core and must be complexed by a suitable chelator such as EDTA (Biemond *et al.*, 1988). However the nature of the reducing substance(s) or the chelator(s) is unknown under physiological conditions. Release of iron is also enhanced at low pH (Watt *et al.*, 1985). On the other hand, others have suggested that the mechanism by which iron is released from Ft is through the constitutive degradation of the protein by lysosomes (Roberts and Bomford, 1988).

The administration of a variety of iron compounds to animals, results in an increase in the Ft content of many tissues (Harrison *et al.*, 1980). Other factors related to cell differentiation/proliferation regulate the transcription of Ft. Little is known about the molecular basis underlying the relationship between cellular transformation and Ft. Lymphoid cells contain different levels of H and L Ft according to their lineage, proliferative status, and anatomical site (Dörner, *et al.*, 1983; Vezzoni *et al.*, 1986). Increased Ft synthesis has been reported to occur during cell differentiation (Fibach *et al.*, 1985). Several studies have shown an association between the development of cancer and

increased serum Ft levels (Moroz *et al.*, 1984). This secreted protein may bind to subpopulations of circulating lymphocytes and ultimately affect their cell-mediated immunity (Moroz *et al.*, 1977).

Mechanisms of regulation of Ft synthesis vary depending on the cell type. In cells in which stored iron is used for intracellular needs, such as proliferating cells, there is a transcriptional control where the mechanism of regulation may depend on mRNA concentration (Cairo *et al.* 1985). It has been shown that there was an increase in the relative concentration of mRNA during the differentiation process indicating either changes in transcription to produce the appropriate Ft to meet the needs of each cell type or changes in mRNA stability (Theil, 1987). It has been shown that the increased Ft mRNA levels in differentiating Friend cells arises from increased transcription rather than decreased mRNA breakdown (Beaumont *et al.*, 1987). Iron also increases Ft transcription in these non-specialized iron storage cells. It has been shown that iron administration causes an increase in transcription of Ft L mRNA (Cairo *et al.*, 1986).

On the other hand, in specialized iron storage cells i.e. hepatocytes, the mechanism of regulation depends more on translational control. Iron administration to rats (Kohgo *et al.*, 1980; Bomford *et al.*, 1981), and to cell cultures (Goto *et al.*, 1983; Rittling and Woodworth, 1984; Rogers and Munro, 1987) stimulates subunit synthesis by an actinomycin-resistant mechanism (Zahringer *et al.*, 1976; Drysdale and Munro, 1966) involving mobilization of L subunit mRNAs from a cytoplasmic pool of inactive messages on to polyribosomes (Aziz and Munro, 1986; Rogers and Munro, 1987).

Ferritin genes for both H and L subunits have a highly conserved 28 base sequence in their 5' untranslated region (5' UTR) that is essential for the translational regulation of the Ft L (Aziz and Munro, 1987) and H mRNAs (Hentze *et al.*, 1987) by iron. Deleting part of the 5' UTR containing the conserved 28 base sequence (Aziz and Munro, 1987) eliminates the response to iron, suggesting that iron-sensitive factor(s) in the cytoplasm may bind to this sequence and regulate the availability of both Ft messages for translation. Leibold and Munro (1988) have identified an 87 Kd cytoplasmic protein interacting with RNA sequence in 5' UTR of Ft H and L subunit mRNA. Similar potential sequences are also present in the 3' untranslated region of the TfR mRNA (see below). The degree of complex formation between mRNA and the cytoplasmic protein has been shown to be affected by treatment of rats or cells with iron. This confirms that intracellular iron levels regulate Ft synthesis by influencing the specific association of this repressor protein to the iron responsive element (IRE) on the Ft H and L subunit mRNA.

Inflammation can also increase Ft synthesis (Konijn *et al.*, 1981). Campbell *et al* (1989) have reported that inflammation causes a shift of Ft mRNA to the polyribosomes in liver and spleen. A possible role of an inflammatory cytokine i.e. tumour necrosis factor in modulating Ft synthesis by inducing Ft gene expression has been suggested (Torti *et al.*, 1988). Moreover Rogers (unpublished cited by Campbell *et al.*, 1989) has shown that exposure of hepatoma cells to IL-1 for 12 h shifts Ft mRNA to polyribosomes, suggesting that this cytokine may be the modulator of the inflammatory reaction by which Ft synthesis is enhanced.

As far as lymphoid cells are concerned, Ft, or a subpopulation of isoFts, may play a role in the regulation of the cellular growth of lymphocytes and the immune response (reviewed by Broxmeyer, 1989) as well as mediating T-cell surveillance (Dörner *et al.*, 1980). Synthesis of Ft by lymphocytes is increased by *in vitro* culturing irrespective of whether a mitogen is present of not (Pattanapanyasat *et al.*, 1987; 1988), suggesting that a mechanism for increasing Ft synthesis by activation may exist that is distinct from iron stimulation.

The biological importance of Ft can be summarized by the following functions, though these functions may be related :

1) Storage of iron in specialized iron storage cells such as hepatocytes and macrophages, for use by other cells. These cells tend to contain the largest amount of Ft, and usually show the largest increase in Ft levels under conditions of iron overload.
2) Storage of iron for subsequent intracellular metabolic use (in cells that are undergoing proliferation, differentiation, and development).
3) Storage of iron for detoxification (in iron overload), which probably constitutes the most evident role of Ft.

1.1.4.2 Haemosiderin

The other iron sequestering protein haemosiderin is the major iron storage protein in iron overload. Hs is a purely lysosomal product which ultrastructurally resembles Ft. The main difference between Ft and Hs is

that the latter is water insoluble, probably due to the lack of an intact protein shell. The structure of Hs is less well defined than that of Ft. It consists of iron containing granules visible in the electron microscope as massive aggregates of electron dense particles of irregular shape (Dickson *et al.*, 1988) resembling Ft iron cores. These particles consist essentially of hydrated polymeric ferric oxide with some phosphate and peptide groups (Weir *et al.*, 1984). It is generally assumed that Hs is a degradation product of Ft following its polymerization (Hoy and Jacobs, 1981). These polymers may be incorporated to lysosomes and converted into Hs. On the other hand, others are still doubtful if Hs is really a product of Ft (St Pierre *et al.*, 1988; Dickson *et al.*, 1988). Iron is much less readily mobilised from Hs than from Ft, and it is generally considered to be an unreactive form of storage iron, which helps in reducing unwanted release of reactive free iron in iron-rich tissues (O'Connell *et al.*, 1986a).

1.1.5 Cellular iron uptake: the transferrin cycle

A primary function of Tf is to donate iron to erythrocyte precursors in the bone marrow, which have a large requirement for synthesis of haemoglobin. Iron is also required by non-erythroid cells for many metabolic needs, though the amounts needed are very small compared with erythroid cells.

1.1.5.1 Structure and regulation of the transferrin receptor

Iron uptake by erythroid and non-erythroid cells is a multistep

process. The chemical properties of iron, its extremely low solubility in aqueous solution, and its high reactivity in forming of active oxygen species, demand that in all steps of its transport from Tf into the cell, it is bound to a ligand. The initial step is the interaction and the attachment of Tf to a specific cell membrane receptor (TfR), which was first noted by Jandl *et al* (1959) on reticulocyte cell membranes. Since then large numbers of studies have been carried out exploring this field. It is now generally agreed that iron is transported from FeTf into the cell by a receptor mediated system.

The TfR is transmembrane glycoprotein (Hamilton, 1979) with a molecular weight of about 180 Kd. The receptor is known to be composed of two disulphide-linked subunits of about 90 Kd each (Seligman *et al.*, 1979; Enns and Sussman, 1981a; 1981b). Each subunit contains glycan chains, which account for about 5% by weight of the receptor (Enns and Sussman, 1981a), and are composed of sialic acid, N-acetyglucosamine, galactose, and palmitic acid (Omary and Trowbridge, 1981). Each subunit can bind one Tf molecule. The structural features of the Tf molecule which are involved in its interaction with the receptor are still not known.

The gene for the human TfR has been identified and cloned (McClelland *et al.*, 1984). Using *in situ* hybridization, it was shown that the gene is mapped on the long arm of chromosome 3 in close proximity to Tf and melano-Tf (p97) genes (Rabin *et al.*, 1985). During its biosynthesis, the human TfR undergoes extensive modifications. Those modifications include asparagine linked glycosylation, dimer formation, intersubunit disulphide bond formation, acylation with

palmitate, and phosphorylation. Examination of the promoter regions of Tf genes and TfR genes indicates some similarities in orientation (Bowman, *et al.*, 1988).

There is a reverse correlation between receptor number and iron supply in cell culture (Rao *et al.*, 1985; Taetle *et al.*, 1985; Ward *et al.*, 1984). The level of intracellular iron regulates the expression of the receptor, as the iron chelating agent desferrioxamine (DFO) increased 2.5-fold the *in vitro* translation of TfR by polyadenylated mRNA isolated from K562 erythroleukaemic cells (Rao *et al.*, 1985), while treatment with ferric ammonium citrate and FeTf produced 25 and 50% reductions, respectively. By using light microscopic and immunochemical methods (Sciot *et al.*, 1987) and immunoelectron microscopy (de Vos *et al.*, 1988), a disappearance of hepatic TfR expression was reported in haemochromatosis when iron overload is severe. It has been shown that DFO treatment of K562 cells causes an early increase of TfR mRNA, while haemin treatment has the opposite effect (Louache *et al.*, 1985). The changes in the level of surface TfR are mainly due to mechanisms that affect TfR mRNA pool. It has been shown that the regulatory domain is located in the 3' untranslated region (3' UTR) (Owen and Kühn, 1987), which Müllner and Kühn (1988) have demonstrated regulates the expression of the TfR through differential degradation of mRNA according to cellular iron needs. While treatment of cells with DFO produces no significant effect on TfR gene transcription nor on the nuclear processing of primary transcripts, cytoplasmic TfR mRNA becomes 20-fold more stable. Full induction of TfR mRNA requires about 15 h. After the addition of iron salts, the TfR mRNA decays with a half life of 1.5 h. These observations suggest that one mechanism for

this regulation may be an iron-dependent control of TfR mRNA stability.

Casey *et al* (1988) have identified 5 potential stem-loop structures resembling the Ft mRNA iron regulatory element (IRE) (see section 1.1.4.1) on the sequence of the 3' untranslated region of human TfR mRNA. When two of these 5 elements were inserted into the 5' untranslated region of an indicator gene transcript, they conferred iron regulation of translation and confirmed the importance of the IRE regions in conferring the property of iron regulation of mRNA. However, with the TfR IRE in this location (5') they found that raising intracellular iron caused increased indicator protein synthesis and lowering intracellular iron caused decreased indicator protein synthesis, a regulation pattern opposite to that seen with the TfR gene. These experiment showed that the IRE location within the mRNA, i.e., 5' or 3', determined the pattern of this iron regulation. It has been shown that the effect of iron on TfR mRNA is exerted through the binding of a cytoplasmic protein to the regulatory region of TfR mRNA, and binding activity is inversely correlated with the level of intracellular iron (Müllner *et al.*, 1989). They proposed that the stem-loop is the substrate of an RNAase that is sterically hindered by the binding of several iron regulatory factor molecules to the adjacent palindromes. This cytoplasmic protein seems to be similar to the one identified by Leibold and Munro (1988) which binds to the untranslated region of the Ft mRNA (see section 1.1.4.1). This IRE-binding protein may therefore be involved in regulation of expression of more than one protein involved in cellular iron metabolism. Rouault *et al* (1988) have reported that the 3' regulatory region of TfR mRNA can compete with the 5' Ft mRNA IRE for the interaction with IRE-binding

protein, resulting in opposing effects on the biosynthetic rate of Ft and the TfR. However, the mechanism of regulation of TfR and Ft may differ according to the cell type. In human macrophages, iron apparently causes an increase in both TfR mRNA and Ft synthesis. Recently, Neupert *et al* (1990) have isolated and purified the IRE-binding protein which binds to specific palindromic elements in the 5' and 3' untranslated sequences of Ft and TfR mRNA respectively and found it to consist of equimolar amounts of two proteins with molecular weights of 95 and 100 Kd when analysed by SDS/PAGE.

The proliferative status also regulates TfR expression. A highly proliferative status of cells is generally associated with a high density of the TfR (Sutherland *et al.*, 1981). Krönke *et al* (1985) reported increased TfR transcription after activation of peripheral T-cells with PHA and phorbol myristate acetate (PMA). It has been shown that the factors that stimulate cell growth increase the expression on the cell surface of TfR (Lombardi *et al.*, 1989). They showed 5-fold reduction in TfR transcripts within 24 h of DMSO treatment and these reductions proceeded loss of surface-expressed TfR. It has been reported that both the proliferation and iron dependent changes in TfR mRNA levels occur mainly post-transcriptionally (Kühn *et al.*, 1989). Iron level and proliferative status appear to act on different mechanisms, as deletion of sequences on the 3' noncoding region of the cDNA abolishes the iron dependent, but not the proliferation dependent regulation (Owen and Kühn, 1987). It has been reported that activated mouse spleen cells show 50-fold increase in TfR mRNA without any induction of TfR gene transcription.

1.1.5.2 Cellular iron uptake from transferrin

The uptake of iron from Tf is not random. Association constants for the binding of Tf to its receptor have been found in the range of 5 x 10^6-2.3 x 10^9 M^{-1} depending on the temperature, pH, type of cell, and the degree of saturation (Hamilton *et al.*, 1979; Young and Aisen, 1980; Ward *et al.*, 1982). It has been shown that there is a marked preference of receptors for diferric over monoferric Tf (Huebers *et al.*, 1984). Using the interaction of different forms of ^{125}I-labelled Tf and rabbit reticulocytes Young *et al* (1984) have found that the association constant for receptor binding is 4.6 x 10^6 M^{-1} for apoTf, 2.5 x 10^7 M^{-1} for monoferric Tf (C-terminal), 2.8 x 10^7 M^{-1} for monoferric (N-terminal), and 1.1 x 10^8 M^{-1} for diferric Tf. It therefore seems to be that the amount of iron taken up depends on the number of each molecular species present.

The effect of pH on the binding of apoTf and diferric Tf to reticulocyte membrane receptors was investigated by Morgan (1983), who found that the binding of apoTf to the cell membrane was of high affinity below pH 6.5. By contrast, diferric Tf showed high binding between pH 7.0 and pH 8.0 with much weaker binding below pH 6.5.

The second step of iron uptake is the internalization of Tf, which is achieved by the general process of receptor mediated endocytosis. Whether phosphorylation-dephosphorylation of the receptor may give a signal for internalization, and play a role in directing receptor traffic from the surface on to the endosome network and back into the surface is a matter of considerable controversy. It has been reported that TfR undergoes protein kinase C-dependent phosphorylation and internalization

in response to treatment by phorbol diesters (Kohno *et al.*, 1985; May and Tyler, 1987; May *et al.*, 1986). FeTf has been found to induce a 10-fold increase in the activity of protein kinase C in CCRF-CEM cells probably through increasing synthesis of the enzyme, while apoTf and iron in the form of Fe-citrate or complexes of Tf with several other metals, did not increase protein kinase activity (Phillips *et al.*, 1987). However, gene transfection experiments have failed to prove a role for receptor phosphorylation in mediating TfR internalization or cycling (Rothenberger *et al.*, 1987; Davis and Meisner, 1987; Zerial *et al.*, 1987; McGraw *et al.*, 1987). Binding of Tf to its receptor generates a lateral mobility of TfR complex to achieve an area of increased numbers of ligand receptor complexes. However there is still a controversy over whether the internalization process is spontaneous and occurs independently of ligand binding (Watts, 1985) or whether it requires Tf bound to the receptor to switch on the process (Klausner *et al.*, 1984a). The internalization process is achieved by a specific microdomain invagination of the cell membrane which is supported by clathrin to form coated-pits. Little is known about the mechanism by which receptors are concentrated selectively in coated-pits prior to internalization. The mechanism might involve passive diffusion of receptors in the membrane and their selective retention in coated-pits (Hopkins, 1985), or direct movement mediated by receptor-protein interactions. Alternatively, the aggregation of receptors could induce the formation of coated-pits. The coated pits are pulled from the cell surface into the cytoplasm through assembly of the clathrin coats. The vacuoles generated rapidly lose their clathrin, producing vesicles which associate and fuse generating the so-called endosomes (Willingham *et al.*, 1984). Internalization is rapid

and an energy requiring process (Karin and Mintz, 1981).

The mildly acidic environment within the endosome of pH 5-6.5 (van Renswoude *et al.*, 1982) protonates the anion (CO_3^{2-} or HCO_3^-) in the binding site adjacent to the iron and provokes iron release (Crichton, 1985). It has been shown that methylamine, ammonium chloride, and chloroquine, which increase vesicular pH, decrease the release of iron from the protein to the cell (Morgan, 1981) without inhibiting internalization of the Tf. Other studies employing mutants of CHO cells, which are defective in endosomal acidification, have demonstrated that such cells cannot remove iron from internalized Tf (Klausner *et al.*, 1984b).

Little Tf is degraded during the process of iron delivery (Karin and Mintz, 1981), and instead of becoming separated apo-Tf remains tightly coupled to the receptor inside the endocytic vesicle, escapes lysosomal degradation, and returns intact together with its receptor to the cell surface by an unidentified mechanism of some kind of reverse endocytosis within a microtubule-associated endosome (Willingham *et al.*, 1984; Willingham and Pastan, 1985). At the surface the apo-Tf dissociates from the receptor into the extracellular milieu, since the apo-Tf-TfR complex is poorly stable at neutral pH. The Tf is then available for many plasma-to-cell cycles, as is indicated by its long plasma half life (7-10 days) compared with its passenger iron (1.5-2 h) (Katz, 1961).

1.1.6 Metabolism and distribution of intracellular iron

Little information is available on the fate of iron within the cell. Once iron is released from the Tf inside the cell, the metal undergoes a series of intracellular transfers. In non-erythroid cells intracellular iron exists in a number of forms that can be summarized as iron containing enzyme systems, stored iron (Ft and Hs), and a "labile pool".

The iron released from Tf may initially enter lysosomes, and it then somehow finds its way across the lysosomal membrane. Despite these uncertainties about the mode of transport of iron to the cytosol, transport of iron across biological membranes usually involves a ferric-ferrous transition (Romslo, 1980). Low molecular weight complexes of Fe^{3+} have been postulated as the species which transport iron through membranes (May and Williams, 1980).

Once in the cytosol, iron passes to an unidentified pool which is dialysable and available for binding by iron chelating agents. The precise identity of the ligands which bind the iron in this pool are not known, but the multiplicity of possible reactions of iron with low molecular weight molecules such as cysteine, glutathione, ascorbic acid, glucose, fructose, and other molecules such as ATP and other nucleotides (Romslo, 1980), has given rise to the concept of an intracellular transit pool, which constitutes a pivot of intracellular iron metabolism and may act as a precursor for functional and storage compounds (Lynch *et al.*, 1974). The idea of a "labile pool" or "transit pool" has emerged from difficulties in explaining intracellular iron metabolism without postulating such an intermediate pool (Jacobs *et al.*, 1977) between the extracellular

(transport) and intracellular (storage iron and iron containing proteins) forms of the metal. In reticulocytes, iron complexes with a molecular weight below 2000 d were detected (Pollack and Campana, 1981; Bakkeren *et al.*, 1985). In mature erythrocytes, GTP and ATP chelated iron were found in an amazing concentration of up to 100 μM (Bartlett, 1976). It has also been shown that rat liver, kidney, heart, brain, spleen, and pig brain, have small but significant amounts of iron in a form available to DFO (Linder *et al.*, 1983).

In reticulocytes, the bulk of intracellular iron is found in haemoglobin. In non-erythroid cells the proportion used for haem synthesis is much less, only 5% being found in haem compounds in PHA-stimulated lymphocytes incubated with Tf for 3 h (Bomford *et al.*, 1986). The major proportion of the intracellular non-haem iron is found in Ft and Hs. After 2 h incubation of isolated rat hepatocytes with ^{59}FeTf almost 70% of iron can be recovered in Ft (Young *et al.*, 1985). The other pool of iron consists of iron incorporated into a number of non-haem iron proteins such as electron transport iron sulphur proteins, oxygenases, aconitase, and ribonucleotide reductase.

Little is known about the mechanisms by which intracellular iron is mobilised from Ft. The presence of apoTf does not appear to be essential for the mobilization of iron from macrophages (Saito *et al.*, 1986). It thus appears that the inverse Tf cycle (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983) would probably not be appropriate to explain the mechanism of intracellular iron mobilization.

CHAPTER ONE

PART TWO

LITERATURE REVIEW OF IRON AND LYMPHOCYTE FUNCTION

1.2.1 The lymphocyte

The function of the immune system is to discriminate between self and non-self, and mount responses to rid the body of potentially life threatening foreign organisms, eg. viruses. In higher vertebrates adaptive immune responses, i.e. responses which give rise to memory, are mediated by lymphocytes. Two main families of lymphocytes can be distinguished, B cells, and T-cells. The T-cells first differentiate into immunocompetent cells by spending a training period in the thymus, while B cells undergo their education in the bone marrow itself. These two interactive subpopulations have many characteristics in common; morphologically, in their habitat, and in sharing a number of cell surface molecules. However they are distinct from each other in their density, their electrophoretic mobility, their half life, their migration after intravenous injection, in a number of cell surface molecules expressed only on T or B cells, and most importantly in the mechanisms of their effector functions.

The immune response occurs most effectively in structured secondary lymphoid tissue. These tissues become populated by cells of reticular origin and by macrophages and lymphocytes derived from bone marrow stem cells. The lymph nodes filter and screen lymph flowing from the body tissues while the spleen filters the blood. Antigen is presented to lymphocytes by macrophages and by specialized antigen-presenting cells in T- and B-cell areas within the lymphoid tissue. B-lymphocytes exhibit antigenic specificity. They proliferate in response to a particular antigen and differentiate into non-proliferating but antibody-

secreting plasma cells. T-lymphocytes also exhibit antigenic specificity, and they also proliferate and differentiate in the presence of antigen, releasing substances called lymphokines, which have important biological properties. While T-cells do not synthesise antibodies, they do fulfill many other immunologically important functions such as helping B-cells to synthesise antibodies, killing tumour cells, and regulating the immune response.

1.2.2 T-Lymphocyte interaction with antigen or mitogen: activation and proliferation

Activation of T-cells involves a shift from a quiescent cell, which is in the G_0 phase of the cell cycle into G_1 and S phase, and the cycle then proceeds through the G_2 phase towards cell division. T-cell activation takes place in sequential stages. Interaction of the antigen or mitogen with its membrane receptor is a crucial first step. Under physiological conditions, such ligand-receptor interaction occurs at the interface of the plasma membranes of an antigen-specific T-cell and an antigen presenting cell (APC) or target cell. These ligand-receptor binding events result in the transduction of these events into intracellular biochemical signals in the form of "second messengers" which influence specific targeted genes, receptive to these signals, that can become transcriptionally active or inactive. The manifestations of T-cell activation include the production of lymphokines, the appearance of new cell surface proteins (which include growth factor receptors), the acquisition of cytolytic effector function, and as a consequence of the production of growth factors and their receptors, proliferation.

Activation of the T-cell induced by an antigen on the surface of an antigen presenting cell must involve an interaction with the T-cell antigen receptor (Ti). This is an immunoglobulin-like heterodimer composed of α- and β-chains which functions as a receptor for combination of antigen and a major histocompatibility complex (MHC)-encoded glycoprotein (Dembic *et al.*, 1986; Bjorkman *et al.*, 1987). An alternative heterodimer TCR containing two different chains i. e. γ and δ has been identified on the surface of a small fraction of T-cells (Chien *et al.*, 1987). The TCR is found on the surface of the majority of mature T-lymphocytes (Aparicio *et al.*, 1989) and may exist as a non-disulphide-linked heterodimer or as a single chain in non-covalent association with a T-cell specific molecular complex composed of at least 5 distinct chains, collectively termed T3 or CD3 to form the (T3/Ti or TCR) complex (Brenner *et al.*, 1985). The function of the TCR is a matter of considerable speculation. A suggested role is as an intermediary that delivers transmembrane signals subsequent to antigen receptor occupancy. This is supported by the findings that antibodies directed against CD3 function as polyclonal agonists which are able to mimic stimulation with antigen leading to early manifestation of activation (O'Flynn *et al.*, 1985; Oettegen *et al.*, 1985), and later to lymphokine production (Chang *et al.*, 1982; van Wauwe *et al.*, 1984), expression of lymphokine receptors (Meuer *et al.*, 1984a; Schwab *et al.*, 1985; Tsoukas *et al.*, 1985; Ledbetter *et al.*, 1986), and proliferation (Van Wauwe *et al.*, 1980).

In addition to its role in antigen-induced T-cell activation, the T3/Ti complex appears to be important in the activation of T-cells by the T-cell-specific mitogenic lectins PHA and Con A. Both lectins bind to

large numbers of T-cell surface glycoproteins (Sitkovsky *et al.*, 1984), Although the cell surface molecules responsible for the ability of these lectins to stimulate T-cells are still unknown, studies with solubilised cell surface proteins have demonstrated that Con A can bind to CD3 but not Ti, whereas PHA can interact with the Ti heterodimer but not to isolated CD3 chains (Kanellopoulos *et al.*, 1985). Evidence supporting the role of the T3/Ti complex in PHA- and Con A-induced T-cell activation is the observation that Jurkat mutant cells which fail to express the T3/Ti complex lose the capacity to produce IL-2 in response to either PHA or Con A (Weiss *et al.*, 1984; Weiss and Stobo, 1984). Moreover, reconstitution of the T3/Ti expression in one of these mutants by transfection resulted in the restoration of the PHA and Con A responsiveness of those cells (Weiss *et al.*, 1986).

CD2, the molecule which functions as sheep erythrocyte-binding protein on the surface of human T-cells and which has as many as 6 distinct epitopes (Martin *et al.*, 1983; Yang *et al.*, 1986) is also involved in the activation of T-lymphocytes and thymocytes, including those lacking T3/Ti antigen receptor complex (Sayre *et al.*, 1987). It has been demonstrated that certain combinations of α-CD2 monoclonal antibodies were mitogenic to T-cells, as measured by proliferation or IL-2 production (Meuer *et al.*, 1984b; Brottier *et al.*, 1985). Individual α-CD2 antibodies are insufficient in inducing T-cell activation. Other reports suggested that α-CD3 and α-CD2 mAb can synergize in inducing proliferation in cultures prepared with highly purified T-cells (Yang *et al.*, 1986).

A crucial number of mitogen molecules must bind to the lymphocyte

surface in such a way as to provide a sufficient surface stimulus. Maximum commitment of lymphocytes to DNA synthesis requires some 18 h exposure to mitogen (Wedner and Parker,1976). Following the binding, a redistribution of the ligand-receptor complex occurs. This interaction leads to rapid changes in the surface membrane, and to "cap" formation, as indicated by reacting lymphocytes with fluorescein-labelled Con A at 37° C (Rao, 1982).

Transduction of a transmembrane signal to T-lymphocyte nuclei via the antigen/mitogen receptors seems to occur via a limited number of biochemical pathways utilized by a variety of cell types, when they transit from a non-growing to a growing phase. The antigen/mitogen receptors on T-cells are linked to a polyphosphoinositide-specific phosphodiesterase (PPI-PDE), such that ligation of these receptors provokes polyphosphoinositide (PI) breakdown and formation of hydrolytic products that function as cellular second messengers (**Fig.1**). The precise nature and functions of the molecule(s) coupling antigen/mitogen receptors on T-cells to the PPI-PDE are still largely undefined. Inositol 1,4,5-triphosphate (IP_3), a water soluble compound, and 1,2-diacyl-glycerol (DG) are released from hydrolysis of phosphatidyl inositol biphosphate (PIP_2) (Imboden and Stobo, 1985), which is found in the cell membrane in minute quantities. These two second messengers trigger two parallel pathways that act in concert to elicit a physiological response. The DG translocates Ca^{2+} phospholipid-dependent protein kinase C (PKC) from the cytosol to the cell membrane and increases its activity by increasing its affinity for Ca^{2+} (Imboden *et al.*, 1985), whereas IP_3 increases cytosolic free Ca^{2+} concentrations by

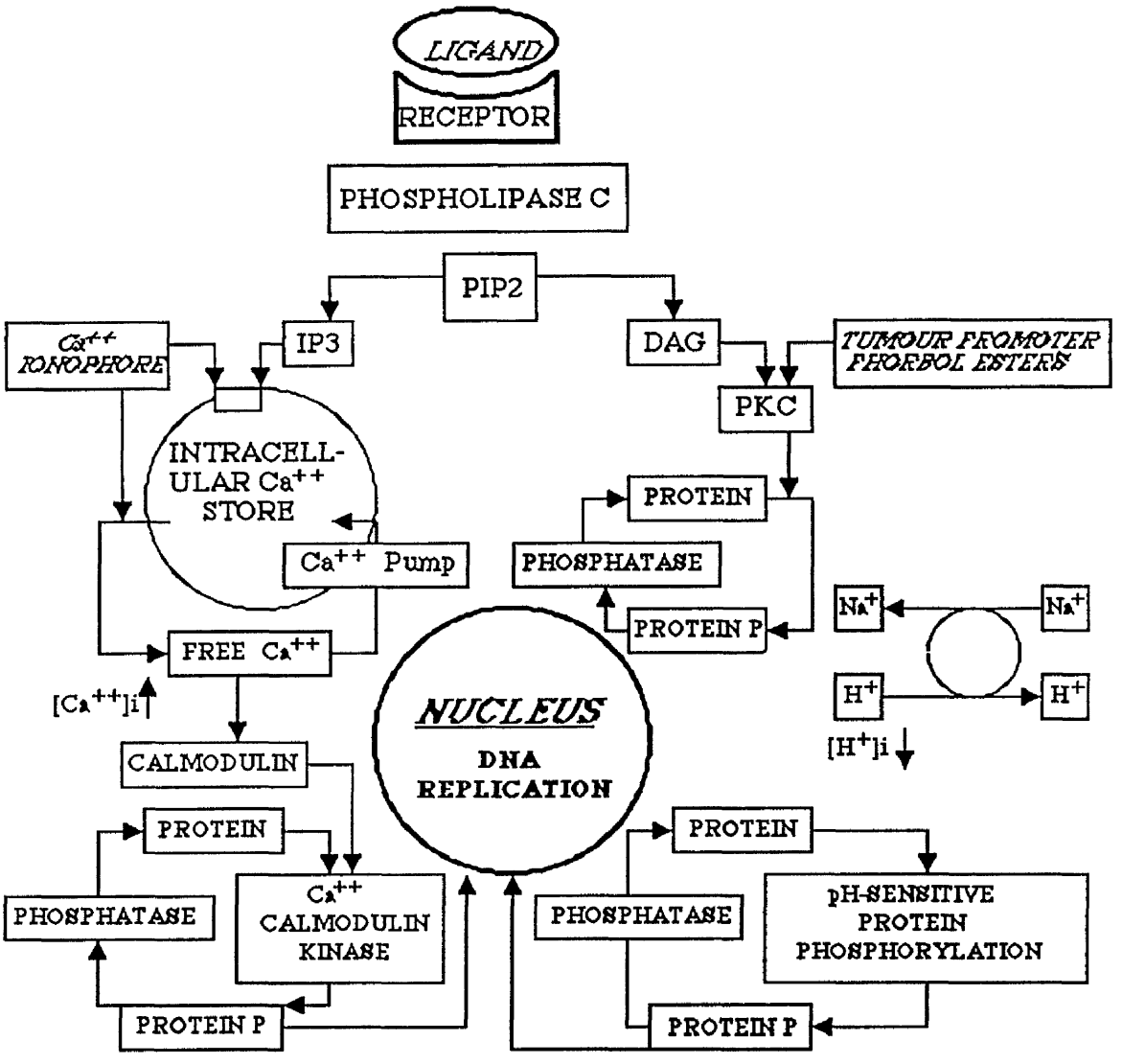

Figure 1 Lymphocyte triggering transmembrane signals. Modified from Isakov *et al* (1986).

causing its release from intracellular stores i.e. mitochondria and endoplasmic reticulum (Berridge and Irvine, 1984) probably by binding to specific receptors within the endoplasmic reticulum (Streb *et al.*, 1983). Calcium ions bind to and thereby activate calmodulin, a cytoplasmic protein which, in the presence of Ca^{2+}, can activate a variety of different enzyme pathways including Ca^{2+}-dependent protein kinases(Cheung, 1980; Means and Dedman, 1980; Berridge and Irvine, 1984). This is followed by a prolonged phase of Ca^{2+} influx through the plasma membrane (June *et al.*, 1986). The increase in Ca^{2+} causes the activation of PKC which mediates phosphorylation of numerous proteins, yet unidentified. Calcium ionophores and phorbol esters can mimic parts of the activation process, whereas neither agent alone is mitogenic. Phorbol ester can bind and activate PKC without noticeable changes in cytosolic free Ca^{2+} concentration (Gelfand *et al.*, 1985). Ca^{2+} ionophore A23187 increases cytosolic free Ca^{2+} influx without influencing PKC activity (Tsein *et al.*, 1982). The combination of Ca^{2+} ionophore and phorbol esters duplicate the role of DG and IP_3 and display strong synergism in inducing the genes for c-*fos*, c-*myc*, IL-2, IL-2R and IFN-γ, and in inducing T-lymphocytes to proliferate (Kumagai *et al.*, 1987, 1988).

As mentioned above, after antigen/mitogen interaction with resting T-cells and the transduction of this interaction into intracellular biochemical signals, these activation signals, in turn, induce the *de novo* expression of certain sets of cellular genes required for T-cell proliferation. These include those encoding lymphokines such as IL-2 and its high affinity cellular receptor. The binding of IL-2 to its high affinity receptors results in their internalization and increases their rate of

removal from the cell surface resulting in a decrease of the level of those surface receptors which are replaced by low affinity receptors (Depper *et al.*, 1985). The subsequent binding of IL-2 to these receptors results in a second wave of gene activation leading to mitogenesis (Robb *et al.*, 1984; Stern and Smith, 1986).

One of these genes activated as a result of the binding of IL-2 to its receptor is the TfR gene (Neckers and Cossman, 1983). This is discussed in detail below.

1.2.3 The role of transferrin in proliferation and iron metabolism of lymphocytes

The proliferation of lymphocytes is a dynamic process which is highly controlled under normal conditions by stimulating, enhancing, and suppressing biomolecule-cell interactions. The iron binding proteins are one group of these molecules.

The importance of iron-containing Tf for the transformation of lymphocytes is well established (Tormey *et al.*, 1972; Dillner-Centrelind *et al.*, 1979; Brock, 1981, Anderson *et al.*, 1982; Brock and Mainou-Fowler, 1983; Brock *et al.*, 1986). The need of these cells to acquire iron is sufficient to account for the Tf requirement, although it has been proposed that Tf may fulfill an additional role unrelated to its iron-donating properties, i.e. its binding to its receptor generates a proliferation signal (Brock and Mainou Fowler, 1983; Seligman,1983). Manger *et al* (1986) have produced an α-TfR monoclonal antibody recognising an epitope different from the Tf binding

site which stimulates the production of IL-2 by HUT 78, a T-cell leukaemic cell line, even in Tf-free medium. Others have demonstrated that Tf exercises its growth factor effect through acting as an electron acceptor for a diferric Tf reductase in cell membranes independently to donating iron to cells (Navas *et al.*, 1986). However, Brock *et al* (1986) have demonstrated that in lymphocyte cultures Tf fulfills its role as a growth factor exclusively as an iron donor.

As regards the question of whether Tf is the obligatory iron donor to proliferating lymphocytes, there are conflicting and contradictory reports. Some authors have shown that the addition of iron without Tf can give good proliferation and differentiation (Rudland *et al.*, 1977; Tanno and Takishima, 1982; Titeux *et al.*, 1984). On the other hand, others have reported that iron itself cannot support proliferation (Phillips and Azari, 1975; Brock, 1981). It has been demonstrated that Tf could be replaced by the lipophilic chelator pyridoxal isonicotinoyl hydrazone (PIH) which can promote proliferation by donating iron via a Tf-independent route (Brock and Stevenson, 1987).

It is generally agreed that DNA synthesis is the main event dependent upon Tf in lymphocyte function, though there may be other cellular metabolic events that are also iron-dependent. Iron uptake was shown to precede DNA synthesis (Brock and Rankin, 1981), because of the need of proliferating cells for iron for the production of the iron containing enzyme ribonucleotide reductase, which is a rate limiting step in lymphocyte proliferation (Hoffbrand *et al.*, 1976; Leberman *et al.*, 1984; Kay and Benzie, 1986). Like other non-proliferating cells, resting lymphocytes express few TfRs. Larrick and Cresswell (1979)

were the first to show that when human peripheral blood lymphocytes were activated by mitogenic lectins such as PHA or Con A, TfR became prominently displayed at cell surface. This observation has been confirmed by many other investigators (Galbraith *et al.*, 1980; Omary *et al.*, 1980; Neckers, 1984). TfR expression is now recognised as one critical event in a complex programme of gene activation induced by mitogenic agents and culminating in progression of resting G_0 phase lymphocytes through G_1 and S phase of the cell cycle (see section 1.1.5.1).

The role of TfR in the iron metabolism of proliferating cells is still not clear, since despite comparable receptor numbers, iron uptake rates by mitogen stimulated lymphocytes are low compared to erythroid precursors (Bomford *et al.*, 1983; Brock and Mainou-Fowler, 1983; Young and Bomford, 1984). It has been suggested that this hyperexpression of TfRs may permit normal immune function to be maintained when Tf saturation is reduced eg. in inflammation or iron deficiency. It has also been reported that the receptor density may vary with the growth phase of the cell (Larrick and Cresswell 1979). Little binding of Tf occurs in resting lymphocytes (Galbraith *et al.*, 1980; Brock and Rankin, 1981; Khalfoun *et al.*, 1986). This indicates that the cells regulate their requirements for iron by the expression of TfR (Iacopetta *et al.*, 1982). Furthermore, interference with TfRs by specific antibodies can affect the cell cycle and growth (Trowbridge *et al.*, 1982). Anti-TfR mAbs that block binding and iron uptake in activated lymphocytes inhibited proliferation, whereas antibodies that bind to TfR but do not block Tf binding are without effect (Mendelsohn *et al.*, 1983; Brock *et al.*, 1986; Taetle *et al.*, 1986; Kemp *et al.*,

1987). Similarly proliferation of murine lymphocytes in medium containing FCS can be blocked by addition of apo-mouse Tf, which presumably binds to the murine TfRs and prevents iron uptake from the bovine Tf in the medium, which has much lower binding affinity to the murine lymphocyte TfR than mouse Tf (Brock *et al.*, 1986). Treatment of mitogen-stimulated lymphocytes with substances that inhibit proliferation such as IFN-α (Besancon *et al.*, 1985), cyclosporine (Prince and John, 1986), or calcium channel-blocking agents (Neckers *et al.*, 1986), also reduce expression of TfRs. The availability of Tf-bound iron in the extracellular medium also influences expression of TfRs; the addition of microbial iron chelators eg. DFO or a high affinity iron chelating agent such as picolinic acid (Pelosi *et al.*, 1986), stimulates the cells to increase the number of TfRs. On the other hand, if the culture medium is supplemented with additional iron the number of TfRs decrease (Ward *et al.*, 1984).

As mentioned above T-lymphocyte proliferation is controlled by a series of premitotic signals generated by interaction of growth factors and their membrane receptors. In normal T-cells, TfR expression is tightly regulated and linked to prior expression of tissue specific growth factor receptors and their interaction with appropriate growth factors. It has been reported that TfR expression in PHA or PHA/tetradecanoyl phorbol acetate-stimulated cells is dependent on the interaction of IL-2 with its receptor (Neckers and Cossman, 1983; Krönke *et al.*, 1985). A short period of interaction of lectin with lymphocytes allows activation of the TfR gene, even in the absence of IL-2, although production of IL-2 greatly amplified TfR expression (Pelosi-Testa *et al.*, 1988). This effect

is exclusive to IL-2 since addition of other recombinant cytokines did not induce TfR expression. However, others have demonstrated that this depends on the route of activation and have described an IL-2-independent pathway for induction of TfR mRNA by stimulating the cells with a combination of phorbol 12, 13 dibutyrate/ionomycin (PDB) (Kumagai, *et al.*, 1988). In this study accumulation of TfR mRNA occurred early in PDB/ionomycin-stimulated T-cells (6 h) in contrast to PHA-stimulated T-cells in which TfR mRNA reached a peak only between 12-24 h. Other studies have been trying to define a temporal sequence of gene activation in human T-cells stimulated with PHA and PMA using nuclear transcription (Krönke *et al.*, 1985). These studies indicated that the proto-oncogene c-*myc* is activated rapidly, reaching peak transcription within 6-9 h. These findings are consistent with the earlier studies of Kelly *et al* (1983) who showed that c-*myc* expression is followed by IL-2R and IFN-γ transcription which peak at 9-15 h. IL-2 gene transcription is also initiated early but does not reach maximal levels until 24 h. Transcription of these four genes was not inhibited by the addition of cycloheximide, indicating that gene expression was not dependent upon the protein products of the other genes. In contrast, the TfR gene was not expressed until 24 h after mitogen activation, and did not reach peak levels until 48 h (Krönke *et al.*, 1985). Addition of cycloheximide at the initiation of culture completely blocked TfR gene transcription, suggesting a dependence on proteins derived from other genes.

Tf is also synthesised in the course of activation by T-helper/inducer lymphocytes. Tf synthesis by these cells appears to be part of a normal autocrine reaction in which growth factors and their

respective receptors act as premitotic signals for DNA synthesis that is followed by cell division and proliferation. It has been shown that the Tf gene is expressed at a specific stage during the premitotic events of T-lymphocyte proliferation, by the $T4^+$ "inducer" subset of lymphocytes (Lum *et al.*, 1986), and that Tf transcription is an intermediate event in the IL-2 autocrine cycle, and occurs after IL-2 transcription and prior to expression of the IL-2R and TfR. The nature and origin of Tf synthesis by lymphocytes as well as its impact on the autocrine pathway of T-lymphocyte proliferation await further study.

1.2.4 Effect of iron deficiency and iron overload on lymphocyte function

Both iron deficiency and iron excess have been shown to alter cellular immune and non-specific defence functions. It has been demonstrated that when the iron saturation of Tf is very low, the rate of proliferation of mitogen stimulated lymphocytes is decreased (Brock, 1981; Phillips and Azari, 1975). It has also been shown that supplementation of lymphocyte cultures with serum from iron-deficient mice does not permit optimal proliferation due to the reduced Tf saturation (Mainou-Fowler and Brock, 1985). Furthermore, It has been demonstrated that splenic lymphocytes as well as purified T- and B-cells from iron deficient mice showed a depressed transformation response to mitogens (Kuvibidila *et al.*, 1983) and decreased cytolytic activity (Baliga *et al.*, 1981). The effect on cellular immune function of excess iron *in vitro* has been studied by a number of investigators. Bryan *et al* (1981) demonstrated that high concentrations of ferric citrate could inhibit

a human mixed lymphocyte reaction. Iron was shown to act on the responder cells rather that the stimulator cells. Keown and Descamps-Latscha (1983) reported that excess iron , in a variety of different forms, could inhibit antigen-induced lymphocyte proliferation and cytotoxic T-lymphocyte (CTL) sensitization, but not CTL effector function. In their studies they used varying concentrations of ferric iron as ferric citrate, ferric nitrate, and also ferric chloride at pH 7.2 where solubility may be a problem. It has also been reported that lymphocytes from iron overloaded rats showed a marked reduction of proliferative capacity after a mitogenic stimulus and a dramatic decrease of their capacity to repair DNA damage (Pietrangelo *et al.*, 1988).

Clinical and experimental iron-overload are associated with a high incidence of infection (Weinberg, 1978; 1984) and neoplasia (Powell *et al.*, 1971). One possible reason for this association is that iron-overload may interfere with normal immune surveillance. In clinical iron overload a small but significant amount of iron is 'free' or non-Tf bound. It has been shown that in iron overload, non-Tf bound iron can be present (Hershko *et al.*, 1978; Batey *et al.*, 1980). It has been demonstrated that non-Tf bound iron Fe^{3+} has an inhibitory effect on the generation of cytotoxic T-lymphocytes by affecting all the major T-lymphocyte subsets. Good *et al* (1986) have found that Fe^{3+} reduced both the cloning efficiency of human $CD4^+$ precursor lymphocytes and the rate of clone growth of the T-cells that did proliferate. The same group has found that low levels of exogenous Fe^{3+} similar to those concentrations reported in the non-Tf bound fraction of the serum of patients with iron-overload, significantly reduced the generation of CTL

by an effect on both regulatory lymphocytes and CTL-precursors.

Spleen cells from iron-overloaded animals had also markedly reduced ability to generate allo-specific CTL, though a frequency analysis of CTL-precursors revealed that these same animals had normal numbers of allo-specific CTL-precursors, suggesting that a regulatory cell defect was responsible for the reduced ability to generate effector cells in the mixed lymphocyte culture (Good *et al.*, 1987). Iron-overload can thus place a limit on the number of available T-helper cells *in vivo* apparently by a reduction in the number of functional helper T-cells. Bryan *et al* (1986) found that iron (Fe^{3+}) at extremely high concentrations suppressed expression of the human T-cell CD4 molecules, on mitogen activated cells. They showed that this was a specific defect, since iron did not alter expression of other markers on activated cells, including CD8, Ia and the thermostable sheep red blood cell receptor.

AIMS OF THESIS

Although iron is needed by lymphocytes for their proper function, the way that iron affects these cells is not fully understood. One particular area in which there is a lack of knowledge is how these cells respond to different forms and levels of iron, and how this affects the way they handle iron. Therefore, the work presented in this thesis has attempted to investigate uptake of iron by mouse lymph node cells donated in different forms and amounts, and relate it to its effect on proliferation. It has also examined where this iron is located inside the cell by looking at the relative incorporation of the metal into different intracellular compartments. One of the reasons for the conflicting results reported on the effect of non-Tf bound iron on proliferation of lymphocytes could be that these cells have the ability to synthesise transferrin. Therefore the possibility that mouse lymph node cells make Tf was also investigated.

Similar studies have also been performed on the effect of iron on human cells in order to see whether these two species respond differently to different conditions of iron. This has also included an investigation of the effect of Lf on lymphocyte proliferation. In particular the role of Lf in promoting proliferation of human lymphocytes by sequestering excess iron has been investigated. Numerous studies have demonstrated a regulatory role in the immune response for Lf, but there are conflicting reports concerning whether this protein fulfills a similar role to Tf. As mentioned in the preceding review it is not clear which human T-cell subsets are primarily affected by high levels of iron. It was therefore

decided to study the effect of different forms and amounts of iron on the expression of T-cell markers to investigate whether different T-cell subsets respond in different ways to iron in varying forms and amounts.

Finally, because it is often stated in the literature that transformed cells generally have reduced needs for nutrients and growth factors compared with their normal counterparts, a similar study was carried out on the T-transformed line CCRF-CEM to examine whether iron was required in similar amounts and forms.

CHAPTER TWO

THE EFFECT OF DIFFERENT LEVELS AND FORMS OF IRON ON MURINE LYMPHOCYTES

2.1 INTRODUCTION

An immune response is a complex process, involving activation and proliferation of lymphocytes, and a complex series of cellular interactions between T- and B-lymphocytes and monocytes, to produce antibodies and to recruit and activate phagocytic and cytotoxic cells. Although direct interactions are probably involved in such responses, soluble factors also play an important role. In addition, other types of factors have been shown to interact with these cells. One such group of factors includes iron and the molecules that bind it, namely Tf, Lf, and Ft. The ability of lymphocytes to proliferate is closely linked with iron, and both inadequate and excessive amounts of the metal can be deleterious (Dallman, 1987; Good *et al.*, 1988). Iron is an important requirement for these metabolically active cells which undergo differentiation and cell division, it being needed in particular for the synthesis of iron containing proteins and enzymes involved in DNA synthesis.

Tf-bound iron appears to be the form in which iron must be supplied (Brock, 1981; Brock and Mainou-Fowler, 1983; Taylor *et al.*, 1987). It has been demonstrated that the ability of Tf to promote lymphocyte proliferation is closely related to its ability to donate iron to the cells (Brock *et al.*, 1986). Little proliferation occurs when apo-Tf is added. Optimal transformation occurs in the presence of Tf which is between 30 and 70% saturated (Brock, 1981). There have been conflicting reports on whether addition of iron in forms other than Tf leads to inhibition or enhancement of the lymphocyte response to mitogens. While some

reported that chelate iron cannot support proliferation (Phillips and Azari, 1975; Brock, 1981; Taylor *et al.*, 1987) others have reported good proliferation (Tanno and Takishima, 1982). Therefore to investigate this matter in more detail, the effect on mouse lymphocyte proliferation of Tf together with two chelators, different in their chemical properties has been investigated. One is a low affinity hydrophilic chelator, nitrilotriacetate, which maintains iron in a soluble form, unlike iron salts, as it is less susceptible to hydrolysis (Bates and Schlabach, 1973). The second chelator, pyridoxal isonicotinoyl hydrazone, is lipophilic and unlike many other chelators was found to be able to donate iron to erythroid precursors for use in haem synthesis (Ponka *et al.*, 1982). Mouse lymph node lymphocytes were chosen as an *in vitro* assay system because they are easily obtainable, and the use of cells from an animal model allows variables which could complicate the interpretation of the results to be eliminated as mice are inbred and age sex etc. are much easier to control. Thus they respond more uniformly than humans.

Much of the knowledge of incorporation of iron into different intracellular iron compartments is derived from studies of erythroid cells. As far as lymphocytes are concerned this area of iron metabolism is almost unexplored. The few studies which looked at this field in cells of the lymphomyeloid series (Bomford *et al.*, 1986; Mattia *et al.*, 1986) have demonstrated that the uptake of iron by the cell is initially to the non-Ft compartment, and this is followed by a time-dependent fractional accumulation of iron into Ft which occurs gradually and increases over a 2-5 h period. However in these studies they cultured the cells for a very short time and looked at cellular distribution of iron at an early time after iron administration, and there are no data concerning intracellular

distribution of iron in cultures over a longer period up till now. Moreover, in the studies mentioned above, FeTf was the exclusive source of iron. Therefore, the work reported in this chapter has attempted to define more closely the effect of different iron forms on lymphocyte transformation *in vitro* in relation to their iron donating ability. This involved the study of the ability of proliferating lymphocytes to acquire iron from Tf and the two iron chelates, i.e FeNTA and FePIH; to investigate the action of these chelating compounds and the effect of their iron content on cellular proliferation, and to see whether iron taken up from these chelates is handled differently from iron acquired from Tf. By looking at the pattern of iron and Ft accumulation and intracellular pathways of iron metabolism, a clearer picture of the effect of these iron carriers on lymphocyte function could be drawn.

One of the reasons for the conflicting results discussed above (section 1.2.3) concerning the ability of non Tf-bound iron to promote lymphocyte transformation, may be explained by the fact that lymphocytes can themselves synthesise Tf which then mediates proliferation. In man activated $CD4^+$ T-cells synthesise Tf (Lum *et al.*, 1986), but in mouse and rat there is so far no convincing evidence of Tf synthesis by lymphocytes. Therefore, Tf synthesis in the mouse lymph node cells upon activation was investigated to confirm that mouse lymphocytes can synthesise Tf as suggested by previous work with human cells (Soltys and Brody, 1970; Nishiya *et al.*, 1980; Broxmeyer *et al.*, 1983; Lum *et al.*, 1986).

In summary therefore, the work reported in this chapter aims to investigate:

1) Uptake of iron by the mouse lymphocyte donated in different forms and amounts in relation to its effect on transformation.

2) Incorporation of iron into different intracellular compartments.

3) The ability of mouse lymph node cells to make Tf.

2.2 MATERIALS

2.2.1 Animals

Balb/C mice of either sex of 16-40 weeks of age were used throughout, and obtained from departmental animal house stock.

2.2.2 Reagents

When necessary, media were prepared in iron-free apparatus i.e. plastic or glassware rendered iron-free by acid washing in 10% HCl overnight then rinsed in deionised distilled water three times.

2.2.2.1 Phosphate Buffered Saline (PBS)

This consisted of NaCl (8 g/l), KCl (0.2 g/l), Na_2HPO_4 (1.15 g/l) and KH_2PO_4 (0.2 g/l) (Analar grade, BDH Chemicals Ltd, Poole, Dorset, England). Solutions were made in deionised distilled water, and when required, aliquots were sterilised by filtration. PBS was made low iron by adding 1% (w/v) $NaHCO_3$.

2.2.2.2 Human Serum Albumin (HSA) solution

A solution of 1% (w/v) HSA (Behringwerke, West Germany) was prepared in RPMI-1640 medium (w/o Hepes; Flow Laboratories, Irvine, Ayrshire, Scotland), was sterilised by filtration and stored at 4° C. The solution was tested by radial immunodiffusion assay (kindly done by Dr S. McGregor) and found not to contain any detectable Tf.

2.2.2.3 Concanavalin A (Con A) solution

A stock solution of Con A (10 µg/ml; Sigma, Dorset, England) was prepared in low iron PBS, sterilised by filtration, dispensed in 100 µl aliquots, and stored at -20° C. Each aliquot was used once only.

2.2.2.4 Ferric nitrilotriacetate (FeNTA)

This was prepared using a molar ratio of 4:1 of NTA to iron to ensure the formation of low molecular weight rather than hydrated polymeric complexes (Spiro *et al.*, 1967). One volume of a freshly prepared solution of $FeCl_3$ (20 mM, BDH) was added dropwise, with constant stirring, to an equal volume of NaNTA (80 mM, pH 7.0; Sigma) and the pH adjusted to 5 with 1 M NaOH to prevent the NTA-free acid precipitating. The resulting concentration of FeNTA was 10 mM (pH 5.0)..

2.2.2.5 Ferric pyridoxal isonicotinoyl hydrazone (FePIH)

A stock solution of 0.3 mg/ml of PIH (Dr P. Ponka, McGill University, Canada) was made in 5-10 ml deionised water, 50 µl of 1 N HCl was added, and the solution was mixed vigorously and left under the hot tap to aid dissolution of the chelator before being filter sterilized.

2.2.2.6 Standard Culture Medium

This was prepared by supplementing RPMI-1640 culture medium (w/o Hepes; Flow) with 0.3 mg/ml L-glutamine (BDH), and 100 IU/ml penicillin and 100 µg/ml streptomycin (Flow). Finally, 10% final concentration of fetal calf serum (FCS) (Flow) was added. For serum

free medium, HSA (1 mg/ml final concentration), 2-mercaptoethanol (2-Me, final concentration 50 μM; BDH), and human Tf (Behringwerke) previously prepared in PBS containing 1% (w/v) $NaHCO_3$ (Analar grade, BDH) to ensure iron removal, usually 50 μg/ml, were added instead of FCS. Different Tf saturations with iron were achieved by adding to Tf solutions appropriate amounts of FeNTA, which is known to maintain iron in the solubilised form and allows it to be readily available for binding to Tf (Bates and Schlabach, 1973).

2.2.2.7 Reagents for α-naphthyl acetate esterase staining

2.2.2.7.1 Fixative. This consisted of formol calcium solution, pH 6.7, containing 10% (v/v) formaldehyde (BDH) and 1% (w/v) $CaCl_2$ in distilled H_2O.

2.2.2.7.2 Phosphate buffer (o.o6 M, pH 5.0). This was prepared by mixing 98.5 ml of KH_2PO_4 (9.08 g/l) and 1.5 ml of $Na_2HPO_4.2H_2O$ (11.88 g/l). The buffer was sterilised by autoclaving.

2.2.2.7.3 Hexazotized pararosaniline. This solution was prepared by mixing equal volumes of two solutions which were prepared as follows:

•*Solution A* This was a freshly prepared solution of 4% (w/v) $NaNO_2$ (BDH).

•*Solution B* This was prepared by dissolving 1 g of Pararosaniline (Waxes & General Laboratories Supplies, London, England) in 20 ml distilled H_2O to which 5 ml of

concentrated HCl was added. The solution was gently warmed to dissolve the pararosaniline, allowed to stand at room temperature to cool, and finally filtered. The filtrate was then stored in the dark at 4° C.

2.2.2.7.4 α-naphthyl acetate esterase stain. This consisted of adding 2.4 ml of hexazotized pararosaniline and 10 mg of a freshly prepared solution of α-naphthyl acetate (Sigma) in 0.4 ml acetone to 40 ml of the phosphate buffer. Finally the pH of the complete reagent was raised to 5.8 with 2N NaOH before being filtered.

2.2.2.8 Reagents for Poly Acrylamide Gel Electrophoresis for ferritin

2.2.2.8.1 Acrylamide solution (40%)

38.96 g Acrylamide (BDH)

1.04 g Bisacrylamide (BDH)

Made up to 100 ml with H_2O, filter sterilized, and kept in cold.

2.2.2.8.2. Sample buffer

50% (v/v) Glycerol (BDH)

0.25 M Tris-HCl (pH 6.8; Bohringer Mannheim GmbH, W.Germany)

0.0125% (w/v) Bromophenol blue (BDH)

2.2.2.8.3 Tank buffer

24 g Tris (Bohringer)

115.2 g Glycine (BDH)

Made up to 4 litre with H_2O

2.2.2.8.4 Fixer

10% (v/v) TCA (BDH)

10% (v/v) Acetic acid (BDH)

30% (v/v) Methanol (BDH)

2.2.2.8.5 Protein stain

0.25% (w/v) Coomassie blue

50% (v/v) Methanol

10% (v/v) Acetic acid

The solution was stirred overnight and filtered before use.

2.2.2.8.6 Perl's Prussian blue reagent

8% (w/v) Ferricyanide mixed 1:1 with 8% (v/v) concentrated HCl (both freshly prepared in double distilled H_2O).

2.2.2.8.7 Destaining solution

20% (v/v) Methanol

10% (v/v)Acetic acid

in H_2O

2.2.2.9 Reagent for immunoprecipitation of transferrin

2.2.2.9.1 3D Buffer

1% (v/v) Triton X-100 (BDH)

0.5% (w/v) deoxycholic acid (BDH)

0.1% (w/v) sodium dodecyl sulphate (SDS; BDH)

0.1 M NaCl

10 mM Na-phosphate buffer, pH 7.5

1 mM Na_2EDTA (BDH)

The buffer solution was sterilized by filtration and kept at room temperature.

2.2.2.10 Reagents for SDS-Poly acrylamide gel electrophoresis for transferrin

2.2.2.10.1 Urea buffer

10% (w/v) SDS

8 M Urea (BDH)

10 mM Tris-HCl, pH 7.0

The buffer solution was sterilized by autoclaving.

2.2.2.10.2 Sample buffer

50% (v/v) glycerol

0.25 M Tris-HCl, pH 6.8

5% (w/v) SDS

5% (v/v) β-mercaptoethanol

0.0125% (w/v) bromophenol blue

2.2.2.10.3 Tank buffer

24 g Tris

115.2 g glycine

4 g SDS

Made up to 4 litre with H_2O.

2.3 METHODS

2.3.1 Preparation of cell suspension and culture conditions

Cell suspensions were prepared from peripheral (brachial, axillary, and inguinal) and mesenteric lymph nodes of the mice. The lymph nodes were removed from freshly killed mice under sterile conditions, and the cells were gently teased out into RPMI-1640 supplemented with penicillin, streptomycin, and L-glutamine as described in section 2.2.2.6. The cell suspension was mixed well, transferred into a sterile plastic universal and left to stand for 1-2 min for any tissue debris to settle out. The cell suspension was then carefully aspirated into a clean plastic universal and the cells were washed twice at 160 *g* for 2-3 min in the culture medium. The suspension contained 85-95% viable cells as measured by eosin exclusion. The cells were finally suspended in the culture medium at a concentration of 2 x 10^6 viable cells/ml. Unless otherwise stated cells were cultured in conical test tubes with Con A at the appropriate concentration. The cells were cultured at 37° C in an atmosphere of 5% CO_2, 95% air for 48 h.

2.3.2 Measurement of proliferation

Cell suspensions were prepared as described above. Proliferation responses were assayed by plating 100 µl aliquots of the cells in a conical bottomed microtitre culture plate (Sterilin) and pulsing with 1 µCi/well of ^{3}H-thymidine (specific activity 52 Ci/mmol; Radiochemical Centre,

Amersham, England) for 4 h before terminating the incubation period. The cells were then harvested on glass fibre mats using a cell harvester (Skatron, Lierbyen, Norway), which were usually left overnight on the bench to dry. Individual discs, each with contents of a single well, were added to 2 ml of scintillation fluid (LKB, Croydon, Surrey, England) and counted for 5 min on a scintillation counter (Packard, Pangbourne, Berkshire, England).

2.3.3 Uptake of iron by proliferating mouse lymphocytes cultured with different transferrin saturations

Uptake of iron was determined by incubating the lymph node cells which were prepared as described in section 2.3.1 with Con A (1 μg/ml) in serum free medium and transferrin at 50 μg/ml saturated to various degrees with ^{59}Fe. This latter was prepared by diluting ^{59}Fe-citrate (specific activity 11 μCi/μg Fe; Amersham) with FeNTA as a source of cold iron. Sufficient iron was added to apo-Tf to give the required saturation at least 2 h before the ferric Tf solutions were added to cultures (Zapolski and Princiotto, 1977), taking into account endogenous iron present in the medium which is in the average of 5 ng/ml (Brock, 1981). After incubation for 2 days in iron-free plastic conical test tubes, the cells were spun down at 1200 rpm for 3 min then washed 3 times with Hanks's solution. To avoid problems of adsorption of isotope to culture tubes, cells were transferred to new tubes after suspension in the third wash solution. Radioactivity associated with supernatant, washes, tubes, and the cells was counted in a gamma counter (Compugamma 1282, LKB Wallac, Croydon, Surrey, England). To determine the

degree of transformation in different samples, 0.5 ml was removed from each cell suspension, and then split into 4 x 100 µl aliquots in a 96 well conical bottomed plate. The cells were pulsed for 4 h with 1 µCi/well of ^{3}H-thymidine, harvested, and counted for 5 min. The radioactive iron was tested and found not to interfere with measurement of uptake of labelled thymidine.

2.3.4 Isolation of ferritin

This was prepared from 100 g of mouse livers following the method reported by Linder and Munro (1972) for preparation of human Ft, except that AcA-22 (LKB, Bromma, Sweden) was used for the gel filtration step. The tissue was minced with scissors and a homogenate was made in four volumes of water using an MSE homogenizer (Crawley, Sussex, England). All materials were kept in an ice bath. The homogenate was heated to 70° C for 10 mins then cooled on ice and centrifuged for 15 min at 2000 g to remove heat-inactivated proteins. The pH of the supernatant was adjusted to 4.8 with glacial acetic acid, left for 4 h, and a further precipitate removed by centrifugation. The pH was adjusted to 7.2 with 1 M K_2HPO_4, solid ammonium sulphate was added to give 50% saturation and the solution was left overnight at 4° C. The precipitate was removed by centrifugation at 2000 g, dissolved in the minimum amount of PBS, and dialysed against 6 changes of the same buffer until the reaction for sulphate with 1% $BaCl_2$ was negative. The sample was fractionated by gel filtration on AcA 22 in a column (1.2 x 90 cm) eluted with PBS, and 1.5 ml fractions collected. The tubes with red colour, indicative of Ft, were pooled and centrifuged at

11 x 10^4 g for 4 h in an L2-65 Beckman centrifuge to sediment the Ft. After redissolving, protein content of the ferritin preparations was measured by the method of Lowry *et al* (1951) using BSA (Sigma) as a standard. Purity was checked by polyacrylamide gel electrophoresis as described in the next section. Mouse spleen Ft was prepared following similar procedure and was kindly provided by Dr X. Alvarez-Hernandez.

2.3.4.1 Agarose Polyacrylamide gel electrophoresis For ferritin

The LKB 2001 Vertical Electrophoresis system was used with a non-denaturing gel consisting of 1% agarose and 4% acrylamide gel, and a 1.5 mm spacer. The agarose solution was prepared by dissolving 345 mg of agarose (Sigma) in 4.37 ml of 1.5 M Tris-HCl, pH 8.8, and 8.5 ml H_2O, and brought to the boil on a hot plate stirrer. When dissolved, the agarose solution was put in a water bath at 50° C. The acrylamide solution was prepared by mixing 4.37 ml of 40% acrylamide (see section 2.2.2.8.1) with 4.37 ml 1.5 M Tris-HCl, pH 8.8, and 8.5 ml H_2O, and put in the same water bath. When both solutions were equilibrated to temperature, they were mixed rapidly and 450 µl of ammonium persulphate (BDH) (freshly prepared) and 25 µl TEMED (Sigma) were added immediately. The mixture was immediately poured into a prewarmed mould with the comb already inserted in it, using a prewarmed glass pipette. The comb was extracted very carefully after the gel had solidified, and the resultant wells were then filled with tank buffer (see section 2.2.2.8.3). The upper reservoir was placed on top of the gel and sufficient tank buffer was added to cover the electrode wire.

The tank was filled with the remaining buffer, and the upper reservoir and gel were placed in the tank. The samples were prepared by adding the Ft solution to the sample buffer (see section 2.2.2.8.2). Two equal volumes of the samples were loaded into two separate wells each containing 20 μg of the protein using long narrow round pipette tips (Bioquone Ltd, Yorkshire, England). The gel was run at 40 mA for approximately 6 h with water cooling. The gel was then incubated for 1 h in fixer solution (see section 2.2.2.8.4), and then cut into two halves each containing one sample, one being stained for protein by incubation in Coomassie blue solution (see section 2.2.2.8.5) for 1 h, and the other for iron by incubation in Perl's Prussian blue reagent (see section 2.2.2.8.6) for 1 h. The gel stained for protein was incubated in the destaining solution (see section 2.2.2.8.7) overnight on a rotary shaker. Both gels were dried by freeze drying using a dryer gel plate connected to a freeze dryer machine. One major and two minor bands were observed all of which stained for iron as well as protein.

2.3.5 Preparation of antibodies against ferritin

The inoculum was prepared by emulsifying complete Freund's adjuvant (Difco) and a solution of MLFt (100 μg/ml) (1:1) to give a final volume of 1 ml in a syringe of the same capacity. To make a stable emulsion the syringe was vibrated on a vortex for 20 min with the plunger and needle secured with tape. The stability of the emulsion was tested by allowing a small drop to fall into a beaker of water, and the emulsion was judged ready to be injected when the drop did not diffuse in the water. The scheme of immunization of rabbits consisted of multiple

subcutanous inoculations in various sites (1 ml vaccine per rabbit) followed by 10 days rest, and then a similar repeat inoculation. After another 10 days a booster was given (50 μg of Ft in 1 ml PBS). After a week, approximately 20 ml blood was collected from the ear vein of each rabbit into a glass universal and allowed to clot at room temperature. The serum was then pooled, transferred to plastic conical-bottomed test-tubes (Sterilin) and centrifuged twice at 3000 rpm for 5 min to remove any erythrocytes. The antibody titre was checked by the ring precipitin test as follows; a small volume of Ft solution (10 μg/ml, 100 μg/ml, and 1 mg/ml) was gently layered on top of a similar volume of antiserum in a Durham tube. The formation of strong precipitin bands with all three concentrations of the protein indicated a very good titre. The antisera were salt fractionated with 33% (w/v) $(NH_4)_2SO_4$ (3 times) (by adding saturated $(NH_4)_2SO_4$ solution, 50% of the original antisera volume). The IgG fraction was dialysed against PBS to remove $(NH_4)_2SO_4$ and stored in aliquots at -20° C.

2.3.6 Affinity chromatography

The following Sepharose 4B affinity columns; Sepharose-normal rabbit immunoglobulins (S-NRIg), Sepharose-rabbit-α-mouse liver Ft (S-Rα-MLFt), Sepharose-normal sheep immunoglobulins (S-NSIg) (NSIg was purchased from Serotec), Sepharose-sheep α-human transferrin (S-Sα-HTf) (Sα-HTf was purchased from Serotec), together with Sepharose-MLFt (for labelling α-MLFt with ^{125}I; see section 2.3.10), were prepared using similar procedures. The required amount of freeze dried CNBr-activated Sepharose 4B (Pharmacia Ltd, Milton Keynes, England) was swollen in 1 mM HCl for 15 min then washed with the same solution on a sintered glass filter (200 ml/g of gel). The

gel was finally washed and resuspended in the coupling buffer (0.1 M Na_2CO_3, pH 7.0 containing 0.5 M NaCl). The proteins to be conjugated were previously dialysed against the coupling buffer for 2 days, the concentration then adjusted to 5-10 mg/ml, as judged by spectroscopic absorption at 280 nm, and finally they were mixed with gel in a ratio of 2:1 (v/v). The mixture was left rotating in an end-over-end mixer for 2 h at room temperature, after which the supernatant was aspirated. Excess ligand was washed away with coupling buffer on a glass filter and any remaining active groups blocked by treatment with 2 volumes of 1 M glycine for 2 h at room temperature. The final product was washed alternately with 3 cycles of 0.1 M sodium acetate buffer (pH 4.0) containing 0.5 M NaCl and the coupling buffer to remove traces of non-covalently adsorbed materials. The conjugates were stored at 4° C in PBS containing 1% (w/v) BSA and 0.02% (w/v) sodium azide. The binding capacity of S-Rα-MSFt and S-Sα-HTf were measured by counting the radioactivity of a known concentration of ^{125}I-Ft and ^{125}I-Tf solution before and after passing each one through a column (1 ml syringe barrel) containing 50 µl of the appropriate packed coupled gel. The capacity of the affinity gel was found to be 5 and 7 µg/µl of settled gel for 2 batches of the S-Rα-MLFt, 4.2 for S-Rα-MSFt and 5.4 µg/µl of settled gel for S-Sα-HTf.

2.3.7 Iron uptake and intracellular distribution of iron in proliferating mouse lymphocytes cultured with different carriers

Intracellular distribution of iron was investigated in proliferating lymphocytes that had been cultured with Con A (1 µg/ml) in serum-free

medium containing different amounts of iron in different forms. Three forms of iron were investigated, ferric transferrin, ferric nitrilotriacetate, and ferric pyridoxal isonicotinoyl hydrazone. The transferrin at 50 μg/ml was used at two different saturations with iron, one low (14%) and the other high (71%). The other iron binding agents were used with iron content equivalent to that of the corresponding iron-containing Tf (10 and 50 ng/ml respectively). The following protocol was adopted:

FeNTA (1 mM) was mixed volume to volume with ^{59}Fe-citrate (specific activity 1331 MBq/mg Fe) and this trace labelled FeNTA was either used as such or added to Tf or PIH. The Fe:chelator ratio was kept constant throughout all the experiments (1:5 for FeNTA and 1:2 for FePIH). The cells (2 x 10^6 viable cells/ml) were cultured in plastic conical test tubes for 48 h, after which they were washed 3 times with Hank's solution, then lysed by adding 1 ml of 1% Triton X-100 in PBS containing 1 mM desferrioxamine (DFO) (CIBA Laboratories, Horsham, England) to the pellet of cells. The addition of DFO at this stage, which reacts with iron to form a highly stable complex, was intended to chelate any available iron at the moment of cell lysis, hence reducing to minimum potential movement of labile iron to other pools as a consequence of cell disruption. The lysates were mixed vigorously and left for 5 min before being centrifuged in a microcentrifuge for 10 min at 10000 rpm, and the precipitates, which consist of insoluble cellular components, after a further washing with Triton X-100/PBS were kept for counting radioactivity. The wash supernatants were added to the original Triton X-100/PBS lysate supernatants. These two together were passed through affinity columns. The affinity columns were made

in a 1 ml syringe barrel containing 50-75 µl of each immunoabsorbent gel. **Fig. 2** shows a diagram of one column. The column contains two specific immunoabsorbents (S-Rα-MLFt and S-Sα-HTf) together with two gels coupled to the immunoglobulins of the corresponding normal sera to correct for non specific binding, i.e. S-NRIg and S-NSIg for S-Rα-MLFt and S-Sα-HTf respectively (see section 2.3.6). The columns were equilibrated and eluted with Triton X-100/PBS, then the lysate supernatants were passed twice through each column to wash out liquid in the dead space. The different parts of the column were carefully dismantled and radioactivity associated with each gel counted in a gamma counter. The filtrates from the immune absorbents were kept for further fractionation into low (<10 Kd) and high (>10 Kd) molecular weight materials using Amicon ultrafiltration cells (Centricon 10 Microconcentrator; Amicon Co, Lexington, Mass., USA) equipped with PM 10 membranes. The Amicon cells were centrifuged in a fixed angle rotor centrifuge (Super Minor, Mk2, MSE) at 1250 *g* for 90 min. The retentates which consist of the high molecular weight fraction consist of ^{59}Fe bound to soluble proteins (other than Ft or Tf). This fraction was called the intermediate molecular weight fraction-^{59}Fe and it probably consists mainly of iron in enzymes and haemprotein. The filtrates containing low molecular weight iron compounds consist of iron bound to DFO.

2.3.8 α-naphthyl acetate esterase (ANAE) staining

A cell suspension of lymph node cells was prepared as described in section 2.3.1. Approximately 6-7 x 10^5 *in vivo* stimulated lymph node

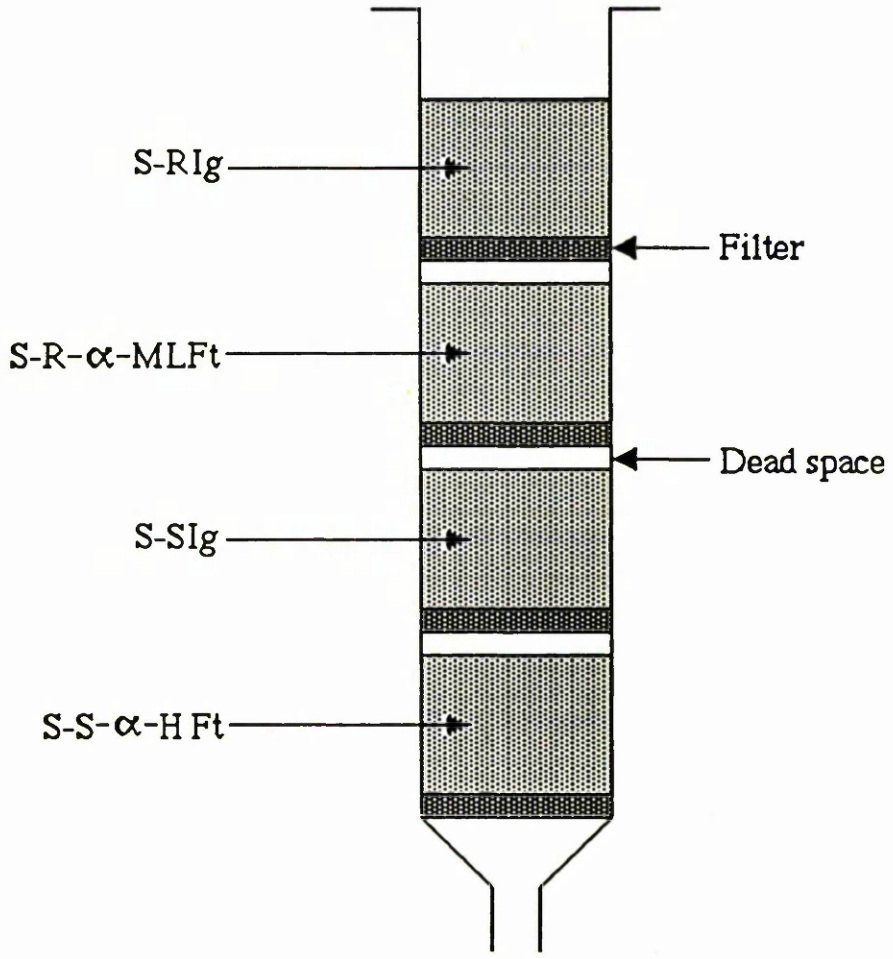

Figure 2 Diagram of an affinity column used in fractionating lysate supernatants for intracellular distribution of iron

cells or lymphocyte depleted cells (see section 2.3.9) were deposited on clean glass slides in a Shandon cytocentrifuge at 1000 rpm for 90 sec and then air dried. Preparations were then fixed in formol calcium (see section 2.2.2.7.1) at 4° C for 10 min and washed in running tap water for 20 min at room temperature. Finally the slides were incubated with the staining reagent (see section 2.2.2.7.4) for 90 min at 37° C. The slides were then washed gently in running water for 10 min, left to dry at room temperature, and then counterstained with 2% methyl green (Hopkins & Williams, Essex, England) for 20 sec. Finally the cells were rinsed in running water, completely air-dried, and mounted with Histomount (National Diagnostics, Somerville, New Jersey, USA). Slides were examined using a Leitz-Welzlar microscope (X 100). T-lymphocytes show small redish brown spots in their cytoplasm, and monocytes and macrophages are characterized by diffuse redish brown staining material throughout the whole cell, while B lymphocyte and null cells do not stain.

2.3.9 Immunoprecipitation method For transferrin

Tf synthesis was assayed in three systems:

1) Normal lymph node cells: Lymph node cells were obtained from six mice as described in section 2.3.1. The cells were preincubated in 5 ml of Tf-free medium consisting of RPMI-1640 (w/o cysteine or cystine; Select-Amine Kit , Gibco Ltd, Paisley, Scotland) in a conical test tube for 1 h at 37° C in an atmosphere of 5% CO_2, 95% air, to allow exocytosis of any endogenous Tf to occur. Cells were then washed twice with PBS, resuspended in 2 ml of RPMI-1640 (w/o cys) containing 100 μCi of ^{35}S-cysteine (specific activity 1021.8 Ci/mmol; DuPont NEN,

Dreireich, W. Germany), and incubated at 37° C in a CO_2 incubator for 5 h. The cells and supernatant were separated, and 9.4 µl of phenylmethyl sulphonyl fluoride (PMSF; 0.21 M in dimethylformamide) and 3ml 3D buffer (see section 2.2.2.9.1) containing 14.4 µl of PMSF were added to supernatant and pellet of cells respectively to give a final concentration of 1 mM. Both supernatant and cells were kept at -70° C until ready for use.

2) Con A stimulated lymph node cells: The cells were cultured at 2 x 10^6 cells/ml in RPMI-1640 containing 10% FCS and 4 µg/ml Con A for 36 h. The cells were then harvested and treated as described above for the non-stimulated cells.

3) In vivo stimulated lymph node cells: Six mice were inoculated into the footpad with 50 µl of an emulsion of PBS/complete Freund's adjuvant (1:1). Three weeks later, the mice were sacrified and the stimulated popliteal nodes were removed. Cells were prepared and then treated as described above for the non-stimulated cells.

Detection of Tf synthesis in adherent and non-adherent *in vivo* stimulated lymph node cells was also performed. After preparing the cell suspension, the *in vivo*-stimulated lymph node cells were resuspended at a concentration of 5 x 10^6 cells/ml in RPMI-1640 complete medium (section 2.2.2.6) containing 10% FCS. The cell suspension was placed in a Falcon 100 x 5 mm plastic Petri dish (5 ml/dish), and incubated for 1 h at 37° C in a CO_2 incubator. For adherent cells, 2 ml of RPMI-1640 (w/o cys) containing ^{35}S-cysteine was added receiving a total of 100 µCi. Non-adherent cells were treated as described above.

Another methodological approach was used to investigate Tf synthesis in lymph node macrophages. This consists of performing an immunodepletion of B and T-lymphocytes before doing the assay. The lymphocytes were eliminated by incubating the *in vivo* stimulated lymph node cells (10^7 cells/ml) with a cocktail of 1:1000 diluted α-mouse IgG (Serotec, Oxford, England), a 1:2000 diluted α-Thy 1 (Serotec), and a 1:10 diluted low-Tox-M rabbit serum complement (Cederlane Laboratories Ltd, Ontario, Canada) for 1 h twice. At the end of the incubation viability was determined using eosin exclusion and was found to be 27-34%. The ANAE stain revealed that the proportion of macrophages increased from 15% in non-depleted *in vivo*-stimulated lymph node cells to 54% in lymphocyte depleted cells. The cells were washed 3 X and counted before being endogenously labelled with ^{35}S-cysteine as described above. Non-depleted *in vivo* stimulated mouse lymph node cells were also assayed as control. The same number of viable cells of both group of cells (depleted and non-depleted) was assayed.

After incubating the cells with ^{35}S-cysteine, the supernatant was split into three 500 µl aliquots in 1.5 ml microvials. Each aliquot was treated as follows:

1) *Test:* To this sample 12 µl of rabbit α-mouse Tf (provided by Dr J.H. Brock) was added.
2) *Competition:* To this sample 40 µg of mouse Tf (provided by Dr J.H. Brock) was added followed by 12 µl of rabbit α-mouse Tf.
3) *Control:* To this sample 8 µl of normal rabbit serum was added.

The samples were incubated for 1 h at room temperature. IgGsorb (The Enzyme Center, Malden, Mass., USA) (100 µl of 60 mg/ml in 3D buffer, freshly prepared each time), protein A coupled to an insoluble base, was then added to all tubes. The tubes were left incubating on an end-over-end rotator at room temperature for 1 h. The IgGsorb was spun down at 6500 rpm on a microfuge for 15 sec. The supernatants were discarded and the IgGsorb was washed 3 times with 750 µl of 3D buffer for 15 sec at 6500 rpm on the microfuge, ensuring that IgGsorb was well suspended between each wash. The washed IgGsorb was resuspended in 70 µl of urea buffer, and incubated at room temperature on an end-over-end rotator for 1 h. The tubes were then spun at full speed (13000 rpm) on the microfuge for 5 min. The pellets were discarded and the supernatants were kept.for an SDS polyacrylamide gel electrophoresis analysis.

2.3.9.1 SDS Polyacrylamide Gel Electrophoresis for transferrin

The standards for the electrophoresis analysis were prepared in separate microvials and 40 µl of urea buffer (see section 2.2.2.10.1) added to each one. The high molecular weight standard mixture (Sigma) (20 µl) was used alongside unlabelled mouse Tf (10 µg) and ^{125}I-labelled human Tf (1000-3000 cpm) (kindly supplied by Miss N. Moughal) to assess the position of the Tf band on the gel and the autoradiograph respectively. To all tubes; samples, and standards, 17 µl of sample buffer (see section 2.2.2.10.2) was added and mixed well. The cap of each tube was pierced with broad-gauge syringe needle and the tubes

heated in boiling water for 5 min

The LKB 2001 Vertical Electrophoresis System was used with 10% agarose gel and 1.5 mm spacers.

The running gel was prepared as follows:

8.75 ml 40% acrylamide (see section 2.2.2.8.1)
8.75 ml 1.5 M Tris-HCl, pH 8.8
16.3 ml H_2O
0.7 ml 10% (w/v) SDS

After mixing well, 450 µl of 10% (w/v) ammonium persulphate (freshly prepared) and 25 µl TEMED were added, mixed by inversion, and the gel solution pipetted into the gel former to a mark made previously at ~1 cm down from the bottom end of the comb. A few ml of water-saturated butanol were added to remove bubbles and ensure a straight surface. When the gel had solidified, the butanol was tipped out and the remaining space washed twice with water. Excess water was removed with a tissue. The stacking gel was prepared as follows:

1 ml 40% acrylamide (see section 2.2.2.8.1)
1.25 ml 0.5 M Tris-HCl, pH 6.8
7.5 ml H_2O
100 µl 10% (w/v) SDS

After mixing 100 µl ammonium persulphate and 10 µl TEMED were added and after inserting the comb, the stacking gel was poured. The comb was extracted very carefully after the gel had solidified. Equal volumes of each sample (~120 µl) were loaded by making up the volume

with tank buffer (see section 2.2.2.10.3). A constant voltage giving a current of 25 mA was applied until the dye entered the running gel, then the voltage was increased to give a current of 45 mA. When front line of the dye was ~1 cm from the bottom edge of the gel, the electrophoresis was stopped, the gel was carefully removed, the stacking gel was scraped off, and the running gel covered with fixer (see section 2.2.2.8.4) in a plastic dish and incubated for 30 min until the dye turned yellow. The gel was then incubated in stain solution (see section 2.2.2.8.5) for 1 h, after which it was incubated overnight with destaining solution (see section 2.2.2.8.7). The gel was rinsed twice with water before being incubated with enhancer (EN^{3}HANCE, New England Nuclear, Boston, Mass., USA) for 1 h. The last incubation was done in water for 30-60 min until the gel was uniformly opaque. The gel was carefully transferred to a piece of blotting paper, placed on a gel dryer plate, and covered with cling film. The plate was connected to a vacuum freezer-dryer for 4-6 h to dry. Autoradiography was carried out using a Kodak XAR-5 film at -70° C for 3-7 days.

2.3.10 Labelling of rabbit antiferritin with ^{125}I

A solid phase procedure of labelling in which the specific antibodies are bound to the antigen coupled to a gel was used to label the specific Rα-MLFt IgG with ^{125}I. This was achieved using the Bolton and Hunter reagent (Amersham International plc., Buckinghamshire, England). The Rα-MLFt was attached to a Sepharose-mouse liver ferritin complex (S-LFt) (see section 2.3.6) to protect its binding site, using the method of Alvarez-Hernandez and Loria (1980) with some modifications

as follows:

An excess of Rα-MLFt (0.5 ml) was passed several times through a small column containing 50 µl of packed S-MLFt complex. The S-MLFt-R-α-MLFt complex was washed 5 times with 0.1 M borate buffer pH 8.5 and left with minimum volume of liquid. The dissolved ^{125}I-Bolton and Hunter reagent was dried under a gentle stream of dry nitrogen gas (the nitrogen was passed through a desiccant tube packed with $CaCl_2$ and silica gel in order to remove any trace of moisture) in a fume hood at room temperature. The S-MLFt-R-α-MLFt was added to the dried ^{125}I-Bolton and Hunter reagent and the vial containing the reaction mixture agitated periodically for 15 min on ice. After reacting the mixture with 0.5 ml of 0.2 M glycine in 0.1 M borate buffer, pH 8.5 for 5 min to block remaining reagent, the complex was removed from the vial and passed through a column of Sephadex G25 (Pharmacia) in a disposable 10 ml plastic pipette (Sterilin) to separate the ^{125}I-labelled α-MLFt from the other labelled products of the conjugation reaction. One ml of potassium thiocyanate (3 M in 0.1 M borate buffer, pH 9.0) was added to the top of the column and elution was started after 5 min to allow the KSCN to enter the gel bed to release the protein from the antibody. Equilibration and elution were carried out with 0.05 M phosphate buffer, pH 7.5 containing 0.25% (w/v) gelatin to minimize the loss of labelled protein by adsorption. Fractions of 1 ml were collected by hand and an aliquot of each was counted. The presence of SCN^- was tested with a drop of 10% (w/v) $FeCl_3$ which gives a dark red colour. The high molecular weight fractions were pooled, dialysed against PBS to remove SCN^-, and stored at -20° C. Similar procedure was used to label Rα-MSFt (the antibody was provided by Dr X. Alvarez-Hernandez).

2.3.11 Immunoradiometric assay for ferritin (IRMA)

The assay for Ft was based on a two site-IRMA (Miles *et al.*, 1974) with some modifications (Alvarez-Hernandez *et al.*, 1981), the principle of which is the binding of a cold antibody to a surface, allowing the antigen to react, and detecting how much is present by a second labelled antibody. This gives a direct dose-response curve.

Preliminary experiments showed that a multiplate (cat No 76-364-05, S-MRC-96 clear, U-shaped wells-clear, Linbro chemical Co., New Haven, Conn., USA) absorbed more protein than other kinds of plastic plates.

2.3.11.1 Titration of first and second antibodies

The titration of the first antibody was done by maintaining constant the amount of the second antibody (4.5 x 10^4 cpm/well) and using known Ft concentrations. The titration of the second antibody was carried out by maintaining the first antibody constant. The aim of these experiments was to find the lowest concentration giving a linear response with an adequate working range. Finally, conditions and the working protocol were chosen as follows:

Samples were prepared by lysing the cells by freeze-thawing in PBS to release intracellular Ft. A 1: 500 dilution (50 µl) of the first antibody made in 0.01 M freshly-prepared sodium carbonate buffer pH

9.2, was incubated overnight to allow adsorption to the wells. Unbound antibody was removed by washing 3 times with 100 µl of PBS. The plate was then incubated for 4 h with 100 µl of 0.5% (w/v) BSA (RIA grade; Sigma) in PBS to prevent non-specific binding of antigen or of the second antibody. It has also been shown that this improves the linearity of the standard curve (Alvarez-Hernandez *et al.*, 1981). Unbound albumin was removed by washing 3 times with 100 µl of PBS. Different concentrations of standard ferritin (0-500 ng/ml) or the unknown samples (50 µl) in at least two different dilutions, were incubated overnight, each one in quadruplicate. All samples were made in PBS containing 1% BSA. Unbound antigen was removed by washing 3 times with 100 µl of PBS. The plates were incubated overnight with 50 µl of a dilution containing approximately 4.5 - 5 x 10^4 cpm of the labelled R-α-MLFt. Unbound antibody was removed by washing with 100 µl of PBS. Finally the wells were cut up with scissors and counted in a gamma counter for 5 min.

2.3.12 Expression of results and statistical analysis

Differences between mean values were analysed by Student's *t*-test and judged significant when *P* values were less than 0.05. Unless otherwise stated the results are presented as typical individual experiments representative of a number of separate experiments which gave similar results.

2.4 RESULTS

2.4.1 Titration of concanavalin A concentrations

Lymph node cell suspensions were prepared as described in section 2.3.1. The cells were cultured in serum-free medium containing 50 μg/ml of 75% saturated Tf, serum-containing medium, or serum-free medium with no addition for 48 h and proliferation responses were assayed as described in section 2.3.2. The concentration of Con A which induced optimum transformation differed from one batch to another. A typical titration pattern is shown in **Fig. 3**. The concentrations of Con A which induced maximum transformation were 0.25 for cells cultured in serum-free media and 2.25 μg/ml for serum containing medium. Although maximum proliferation in cells cultured in serum free medium with or without Tf was obtained at the same concentration of the mitogen, the proliferative response of cells lacking Tf was very much lower than when Tf was present in the culture medium. A new batch of the mitogen was required during the course of this work. Before use it was titrated and gave 1 and 4 μg/ml for serum-free medium and serum containing medium respectively (not shown). Therefore, in the subsequent experiments the concentrations of Con A used were 0.25 or 1 μg/ml for serum-free cultures and 2.5 or 4 μg/ml for serum-containing cultures.according to the batch used.

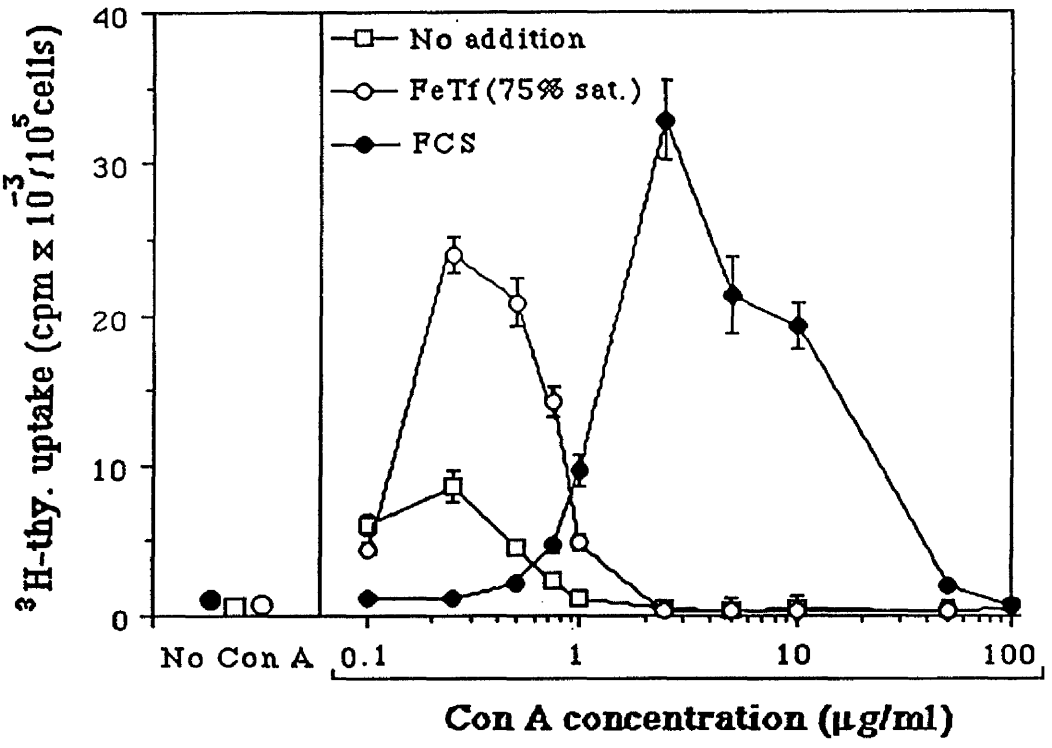

Figure 3 Titration of Con A concentrations in stimulating mouse lymphocyte.
mean + SD (n = 4)

2.4.2 Uptake of iron and thymidine by proliferating mouse lymphocytes cultured with transferrin at different iron saturations

Cells were cultured with Con A (1 μg/ml) in the presence of different iron saturations of Tf ranging from 30% to 120% for 48 h, and the iron associated with the cells was measured as described in section 2.3.3. From **Fig. 4** it is seen that iron uptake increased with increasing Tf saturation up to 90% with about 10% of iron being taken up. Beyond this level, when the amount of iron present added as FeNTA exceeded the binding capacity of Tf up to the equivalent of 120% saturation, the iron uptake increased more rapidly with more than 15% of iron present being taken up. To investigate whether iron uptake from higher levels of saturation occurred at the same rate of increase or if there is a limit at a certain level above which the cells cannot taken up more iron, a new experiment of iron uptake with higher concentrations of iron up to the equivalent of 240% saturation of Tf was performed. Proliferation was assayed in parallel as described in section 2.3.2. The results (**Fig. 5**) show that iron uptake increased at about the same rate up to 120% saturation, as found in the previous experiment. With a further increase in iron present in the cell culture up to the equivalent of 180 and 240% saturation of Tf, the rate of increase in iron uptake rose sharply with 35 and 42% of iron being taken up respectively, indicating that this latter extracellular level of iron (168 ng/ml Fe) did not lead to cellular iron saturation. The two graphs (**Fig. 4** and **5**) of iron uptake versus Tf saturation show that the proportion of iron taken up to iron present in the medium was roughly constant up to 45% saturation of Tf at 5.2-6.7% of

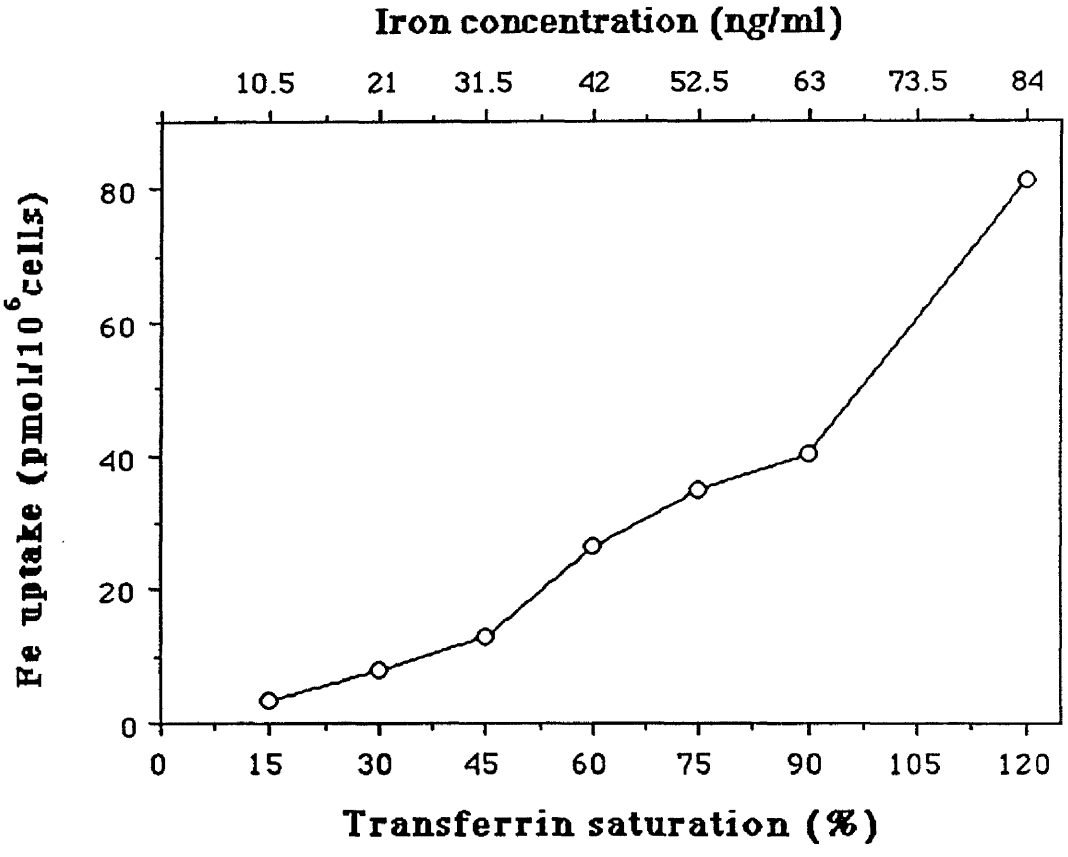

Figure 4 Iron uptake by Con A-stimulated mouse lymphocytes from transferrin of different iron saturations (representative of 5 individual experiments)

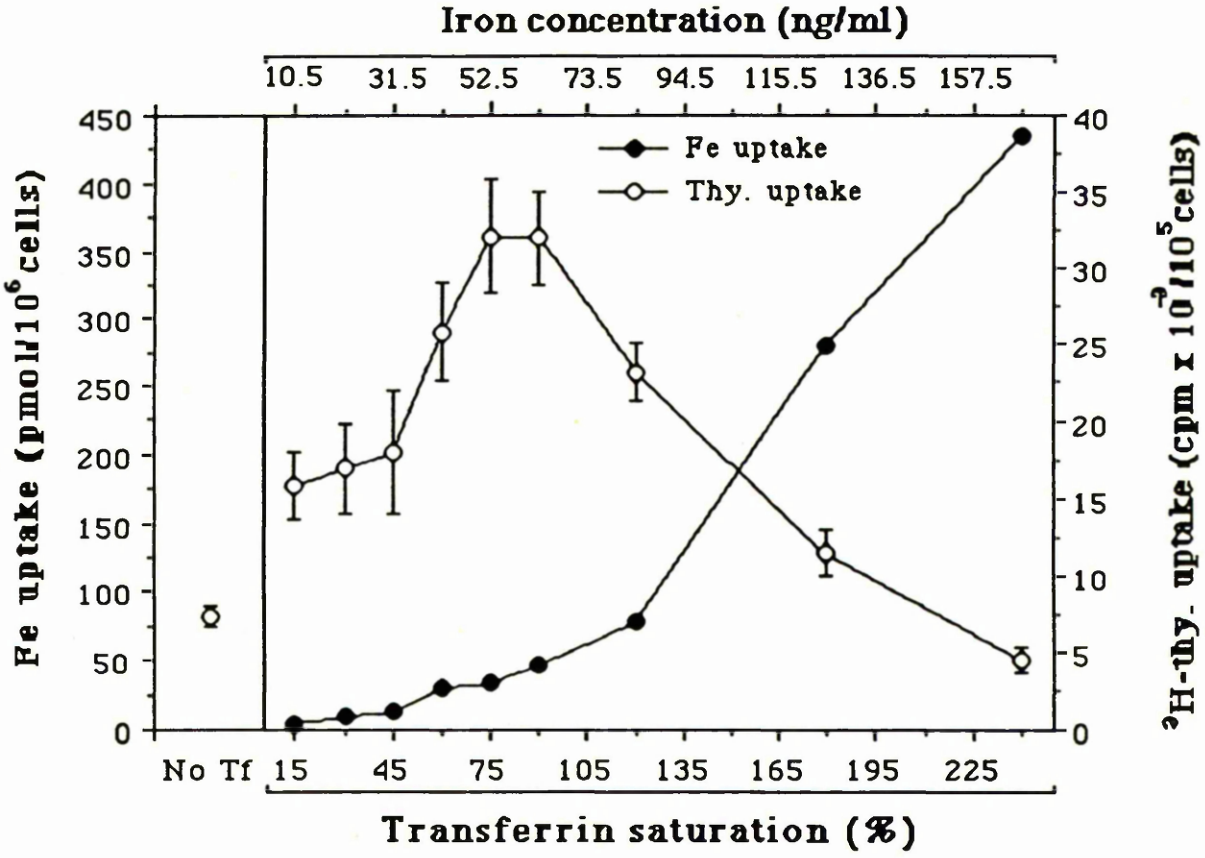

Figure 5 Iron and thymidine uptake by Con A-stimulated mouse lymphocytes cultured with different saturations of transferrin with iron (representative of 4 different experiments). Open points and vertical bars represent mean counts ±SD (n = 4)

iron present in the medium being taken up. When this level of saturation was exceeded up to around the complete saturation level of Tf, this proportion markedly increased. Within this range of Tf saturation (45%-90%) this proportion was 10.3-11.5% of iron present in the medium being taken up. However, beyond the level of complete saturation of the protein, this proportion was directly proportional to the amount of iron present in the medium.

Proliferation, assayed in parallel showed that at lower iron saturations (15-45%), the proliferative response of the lymphocytes was significantly lower than when Tf was fully saturated with iron. An increase in proliferation was seen as the saturation was increased up to about complete saturation. Thereafter, there was a sharp decline of proliferation once this saturation level was exceeded, and proliferation was reduced to below control level when the saturation of Tf exceeded 200%.

The results obtained here at higher iron saturation of Tf (around the complete saturation) differed from earlier findings (Brock, 1981) in which it was found that optimal proliferation occurred with 30-70% saturation of Tf. In view of this, a further assay of proliferation was performed using larger numbers of different concentrations of iron ranging from 0 to 240% saturation. The results (**Fig. 6**) were similar to the previous experiment (**Fig. 5**) with a proliferation peak around 100% saturation of Tf followed by a decrease. Iron free (apo-)Tf was also assayed in this experiment and found to cause a slight but significant increase compared with cells incubated without Tf ($P<0.001$), perhaps due to slight iron contamination in the culture medium.

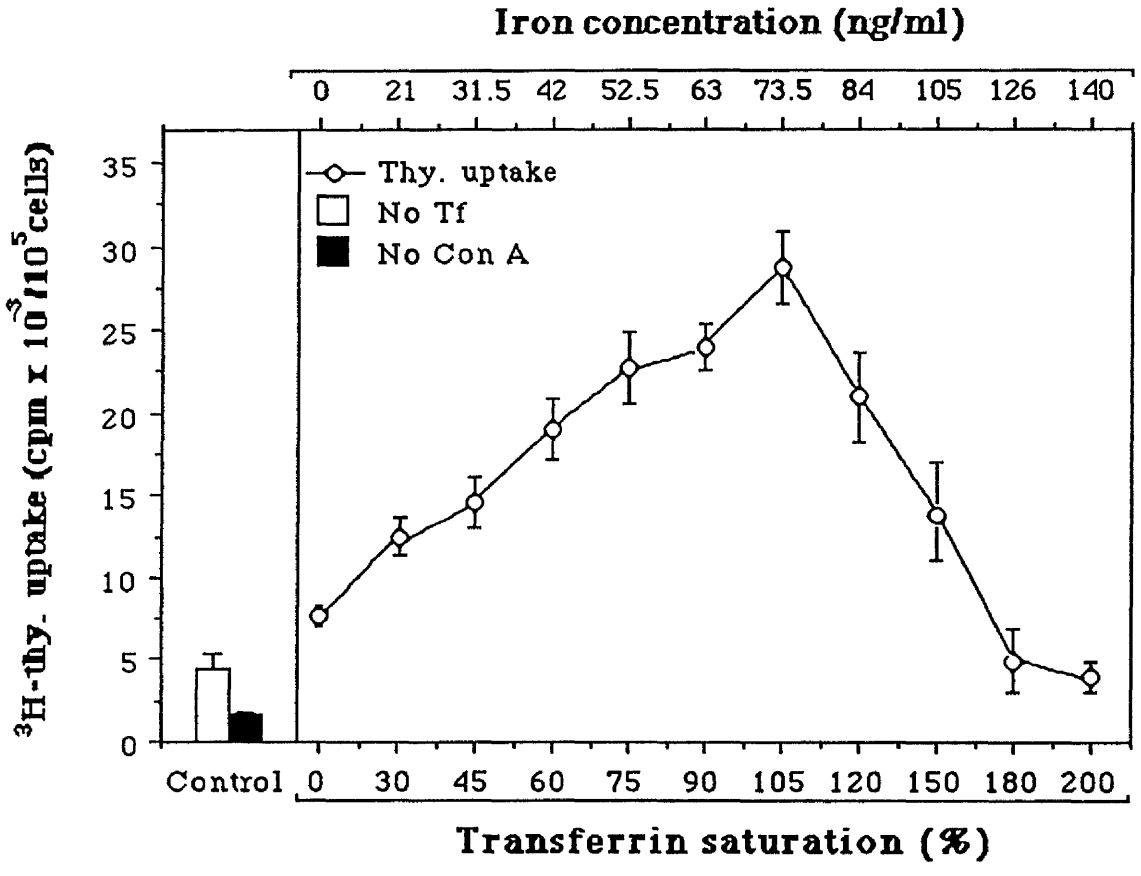

Figure 6 Thymidine uptake by Con A-stimulated mouse lymphocytes cultured with different saturations of transferrin with iron (typical experiment representative of 4 individual experiments) Points and vertical bars represent mean counts ± SD (n = 4).

2.4.3 Proliferation of lymphocytes cultured with different iron chelates

The effect of two different forms of iron on proliferation of Con A-stimulated lymph node cells was examined. The two chelators were FeNTA, the chelator used to load Tf throughout all the experiments where FeTf was used and a lipophilic iron chelator FePIH. The cells were cultured as described in section 2.3.1 in the presence of increasing FeNTA concentrations. Proliferation was assessed as described in section 2.3.2 to investigate whether adding FeNTA alone inhibited proliferation in the same way as it did when iron loaded Tf was present. **Fig. 7**, which shows thymidine uptake versus iron content of the medium indicates that at very low iron concentration 10 ng/ml, the chelator FeNTA did not have any significant effect compared to control cultures with no addition. At higher iron concentrations a progressive inhibitory effect was seen, and at 60 ng/ml, proliferation was completely abolished.

Fig. 8 shows the effect of FePIH on lymphocyte proliferation. The ratio of Fe:PIH was kept constant at 1:2 throughout all the experiments. In contrast to FeNTA, FePIH was effective in promoting proliferation of Con A-stimulated mouse lymphocytes. At low concentration of iron (<40 ng/ml), the complex FePIH was not effective, but when iron concentration was increased in the medium up to 160 ng/ml, proliferation increased and optimum proliferation occurred. The proliferation at this concentration was as good as that with 90% saturated FeTf (63 ng/ml iron), which was used as a positive control.

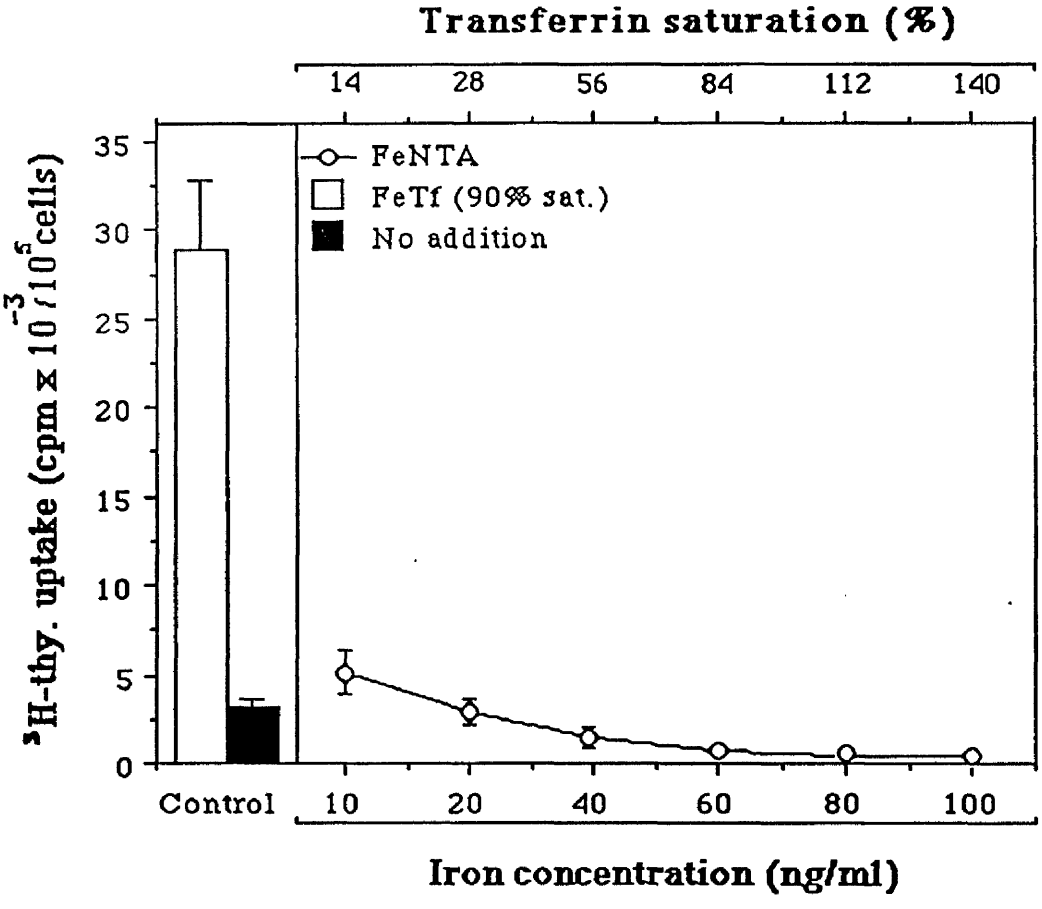

Figure 7 The effect of FeNTA on proliferation of Con A-stimulated mouse lymphocytes (typical experiment representative of 3 individual experiments). Points, columns and bars represent mean counts ± SD (n = 4).

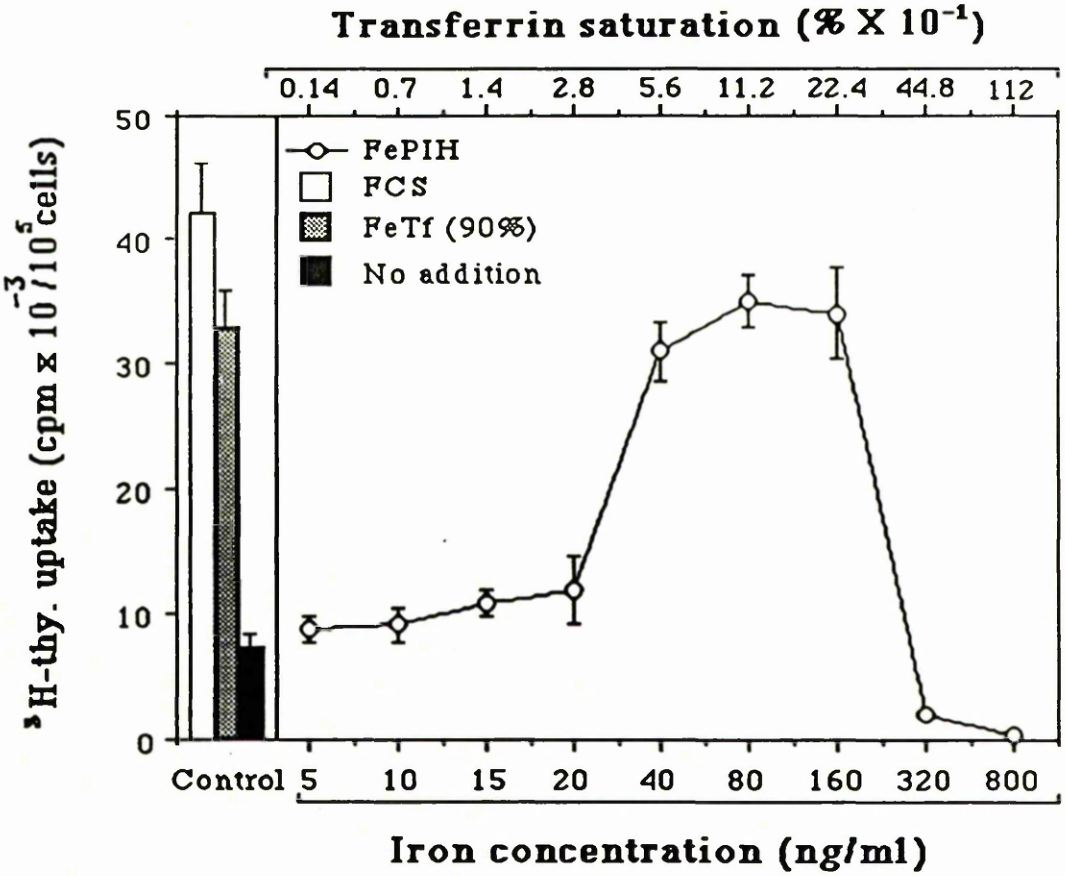

Figure 8 The effect of FePIH on proliferation of Con A-stimulated lymphocytes (results of a typical experiment representative of 4 separate experiments). Points, columns and vertical bars represent mean counts ± SD (n = 4).

Higher concentrations were inhibitory.

2.4.4 Iron uptake and its intracellular distribution in proliferating mouse lymphocytes cultured with different iron carriers

In order to determine the fate of the iron taken up by proliferating lymphocytes from different sources, uptake of radioactive iron delivered by FeTf, FeNTA, and FePIH into the proliferating Con A-stimulated lymphocytes and the subsequent distribution of the metal among intracellular iron compounds was carried out. The cells were lysed in the presence of DFO and fractions were separated as described in section 2.3.7. In the absence of Tf total uptake of iron from both chelates at both concentrations of iron tested was greater than the amount of iron taken up from Tf by the cells (**Fig. 9**). Nevertheless iron taken up from FePIH, was much lower than that taken up from FeNTA at both iron concentrations tested. At 10 ng/ml, iron taken up by cells from FeNTA was some 4.5-fold the amount taken up from Tf, while at 50 ng/ml of iron, the amount of iron taken up from this chelate was 9 times the amount taken up from Tf, 40% of iron present in the medium being taken up by the cells in the former case. Iron donation to the cells by the other chelate FePIH, was about 1.5-fold higher than the amount of iron taken up from Tf at both concentrations tested.

Since there appeared to be differences in iron delivery by the three carriers to cells, it was of interest to determine the relative intracellular distribution of iron taken up from these forms of iron. This was carried out as described in section 2.3.7 which allowed intracellular iron to be

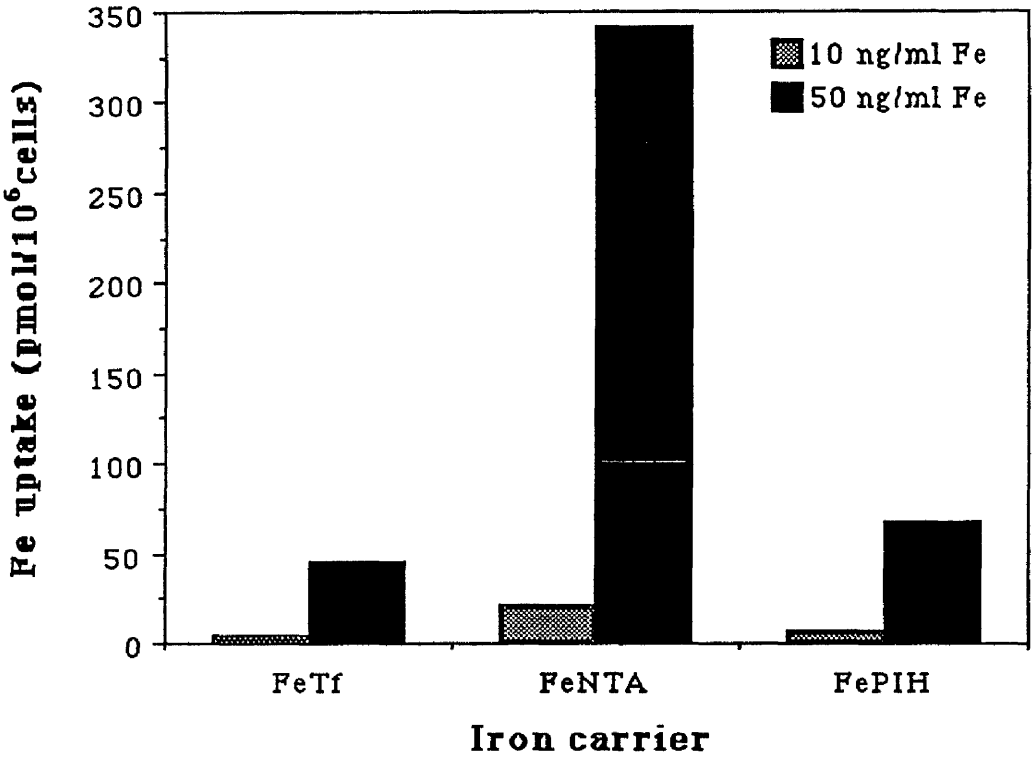

Figure 9 Iron uptake by Con A-stimulated mouse lymphocytes cultured with different iron carriers (representative of 4 separate experiments)

assigned to five different compartments consisting of insoluble materials, iron-bound Ft, iron-bound Tf, iron bound to non-Ft non-Tf macromolecule soluble compounds, and iron capable of being bound to DFO.

The major proportion of iron taken up from the iron chelate FeNTA was found in the insoluble fraction which contains cellular debris and probably haemosiderin. This was true at both concentrations of iron tested (**Fig. 10** and **11**). However, when Tf was present this fraction represented a much lower proportion compared with cells cultured with FeNTA. This was also the case for cells cultured with FePIH. This suggests that much of the iron taken up from FeNTA is not used for cellular metabolism. The chelatable fraction gave fairly similar figures as far as FeTf and FeNTA are concerned. However, with FePIH the proportion of this fraction was slightly higher compared with the proportion found with the two other carriers, at both concentrations of iron. As the iron concentration in the medium was increased the proportion of the low molecular weight fraction showed a noticeable increase with all carriers. However, in absolute figures this fraction was much higher with FeNTA compared with the amount found in this fraction with the two other carriers.

The proportion of intermediate molecular weight-associated iron, defined as soluble non-Ft non-Tf and non chelatable fraction, which probably represents iron being incorporated into enzymes and haemproteins, was much larger in the cells cultured with Tf compared with FeNTA, as it was also in cells cultured with FePIH, especially at 50 ng/ml iron. This indicates that these cells have an increased need for

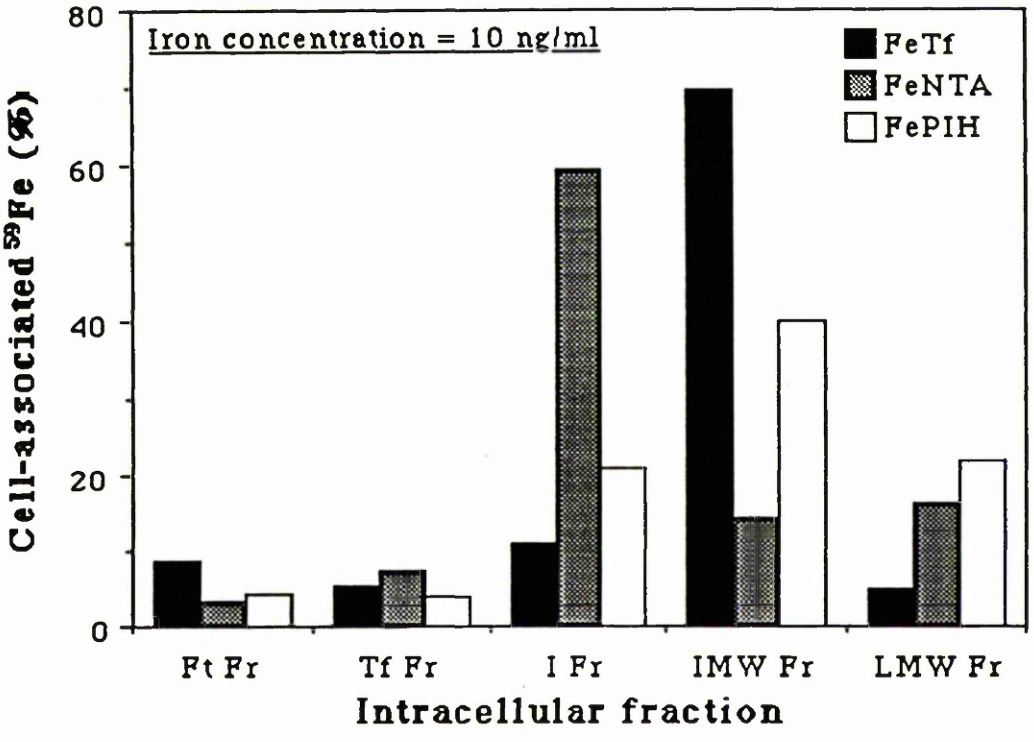

Figure 10 Intracellular distribution of iron in Con A-stimulated mouse lymphocytes cultured with different iron carriers at 10 ng/ml iron (representative of 4 separate experiments)

Ft Fr:	Ferritin Fraction
Tf Fr:	Transferrin Fraction
I Fr:	Insoluble Fraction
IMW Fr:	Intermediate Molecular Weight Fraction
LMW Fr:	Low Molecular Weight Fraction

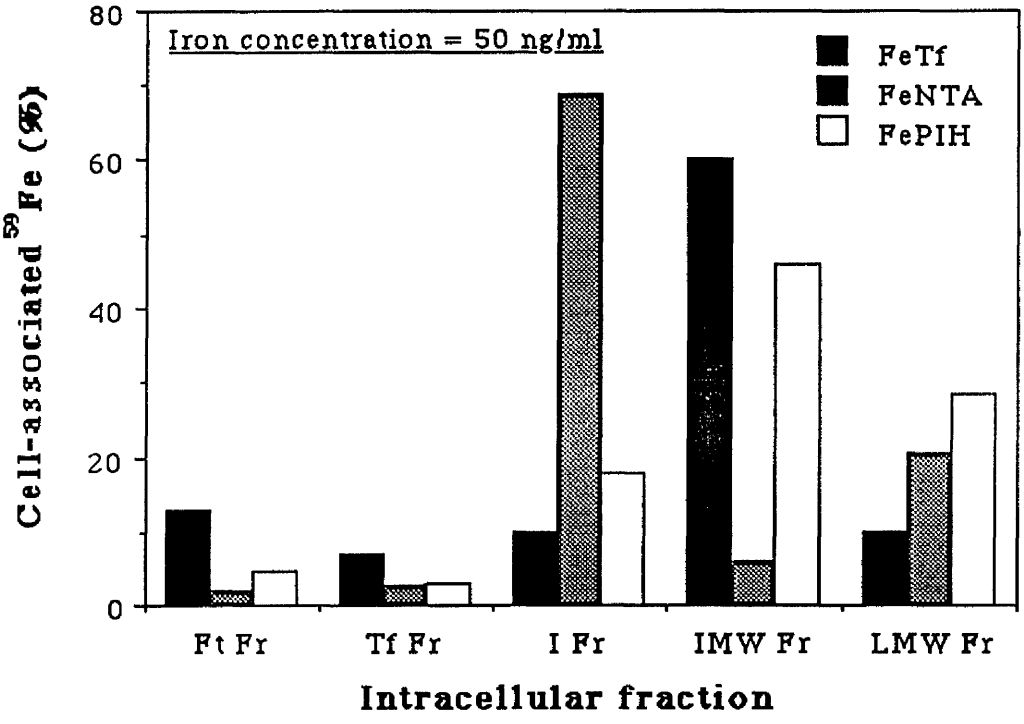

Figure 11 Intracellular distribution of iron in Con A-stimulated mouse lymphocytes cultured with different iron carriers at 50 ng/ml Fe (representative of 4 seprate experiments)

Ft Fr:	Ferritin Fraction
Tf Fr:	Transferrin Fraction
I Fr:	Insoluble Fraction
IMWFr:	Intermediate Molecular Weight Fraction
LMWFr:	Low Molecular Weight Fraction

metabolically active iron. This was not due to the findings that these cells take less overall, as this fraction was greater in absolute figures than when FeNTA was present.

The ferritin iron fraction formed only a small proportion with all iron carriers, particularly with the chelators.

Iron bound to Tf was found in small amounts in the cells incubated with chelates in absence of any exogenous Tf. This could be due to cross reaction between HTf and MTf with α-HTf which was used to fractionate iron-bound Tf. This Tf might be synthesised by proliferating mouse lymph node cells as it has been reported that human lymphocytes can synthesise Tf in the course of activation (Lum et al., 1986). To investigate whether such cross reaction did occur, 1 mg of ^{59}Fe- labelled 50% saturated MTf was passed through two affinity columns, one containing 25 µl of S-Sα-HTf and the other column containing 25 µl of S-NSIgs, to correct for non-specific binding. It was found that 60% of the radioactivity was retained in S-S-α-HTf (**Table 1**), with low non-specific binding (3%), which confirms that there is a cross reaction between human and mouse Tf.

In view of these results, it was decided to investigate Tf synthesis in mouse lymph node cells using an immunoprecipitation method.

2.4.5 Detection of Transferrin synthesis by stimulated mouse lymph node cells

In order to identify Tf synthesis by lymph node cells, the proteins secreted by the cell were endogenously labelled with ^{35}S-cysteine and a

Table 1 Binding of ^{59}Fe-mouse transferrin to α-human transferrin affinity column

^{59}Fe binding to affinity column (cpm x 10^3)			
column I		column II	
S-S-α-HTf	Filtrate	S-S Ig	Filtrate
972	644	43	1443

S-S-α-HTf: Sepharose-sheep antihuman transferrin
S-S-Ig: Sepharose-sheep immunoglobulins

Tf-immunoprecipitation performed on the supernatant, which was then analysed in an SDS-polyacrylamide gel. Tf synthesis was tested in three systems; in unstimulated, Con A-stimulated, and *in vivo*-stimulated mouse lymph node cells as described in section 2.3.9. In *in vivo*-stimulated cells a major band of immunoreactive radiolabelled material similar to mouse Tf was present which was barely detectable when excess cold Tf was added to the supernatant before α-mouse Tf in a competition test or when non-immune serum was used (**Fig. 12**). In Con A-stimulated cells, the supernatant showed an extremely faint band, giving a suggestion that there is a less pronounced synthesis of Tf in those cells compared to the cells stimulated *in vivo*. In contrast, in unstimulated cells Tf was not produced in detectable amounts as there was an equally faint band in all three lines.

Lymph nodes contain together with lymphocytes, other cell types i.e. endothelial cells and macrophages. The number of the latter increased sharply within the node after *in vivo* stimulation as judged by ANAE staining (**Fig. 13**). Thus it is possible that the Tf synthesis seen in *in vivo* stimulated lymph node cells could come from cells other than lymphocytes. It was therefore necessary to investigate Tf synthesis in adherent and non-adherent lymph node cells as described in section 2.3.9. **Fig. 14** shows that both groups of cells synthesise Tf upon stimulation. The band corresponding to Tf synthesised by adherent cells (test line) was stronger than the one corresponding to Tf synthesised by lymphocytes. This estimate depends on the efficiency of the separation step of adherent and non-adherent cells. Therefore, to be more accurate, it was decided to use a more specific method consisting of an

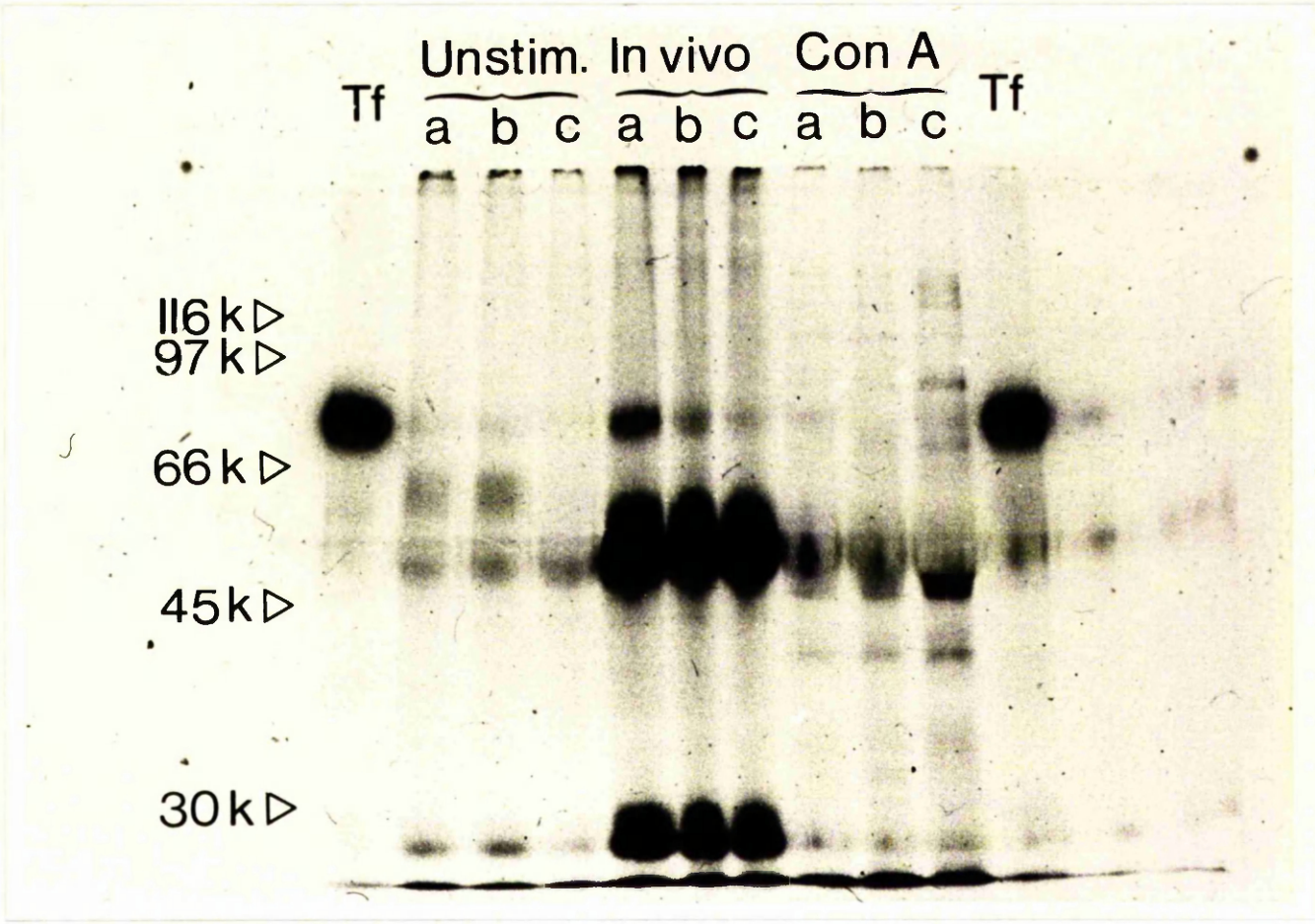

Figure 12 Autoradiography of ^{35}S-cysteine incorporated into transferrin by unstimulated, Con A-stimulated and *in vivo*-stimulated lymph node cells

a: *Test* (+rabbit α-mouse transferrin)
b: *Competition* (+ mouse transferrin then rabbit α-mouse transferrin)
c: *Control* (+ normal rabbit serum)

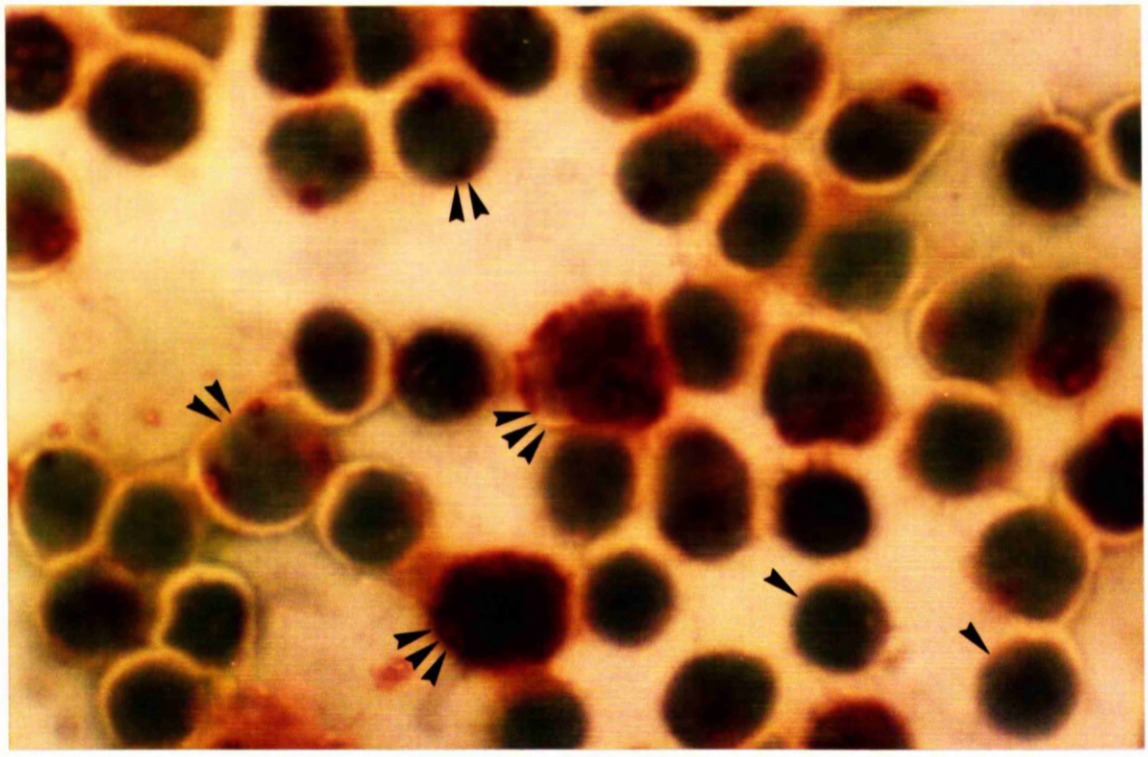

Figure 13 *In vivo*-stimulated lymph node cells stained with α-naphthyl acetate esterase satin. Cells indicated by one narrow are negative cells (B-lymphocytes or null cells), double arrows point to T-lymphocytes and triple arrows to monocytes or macrophages (X 1000).

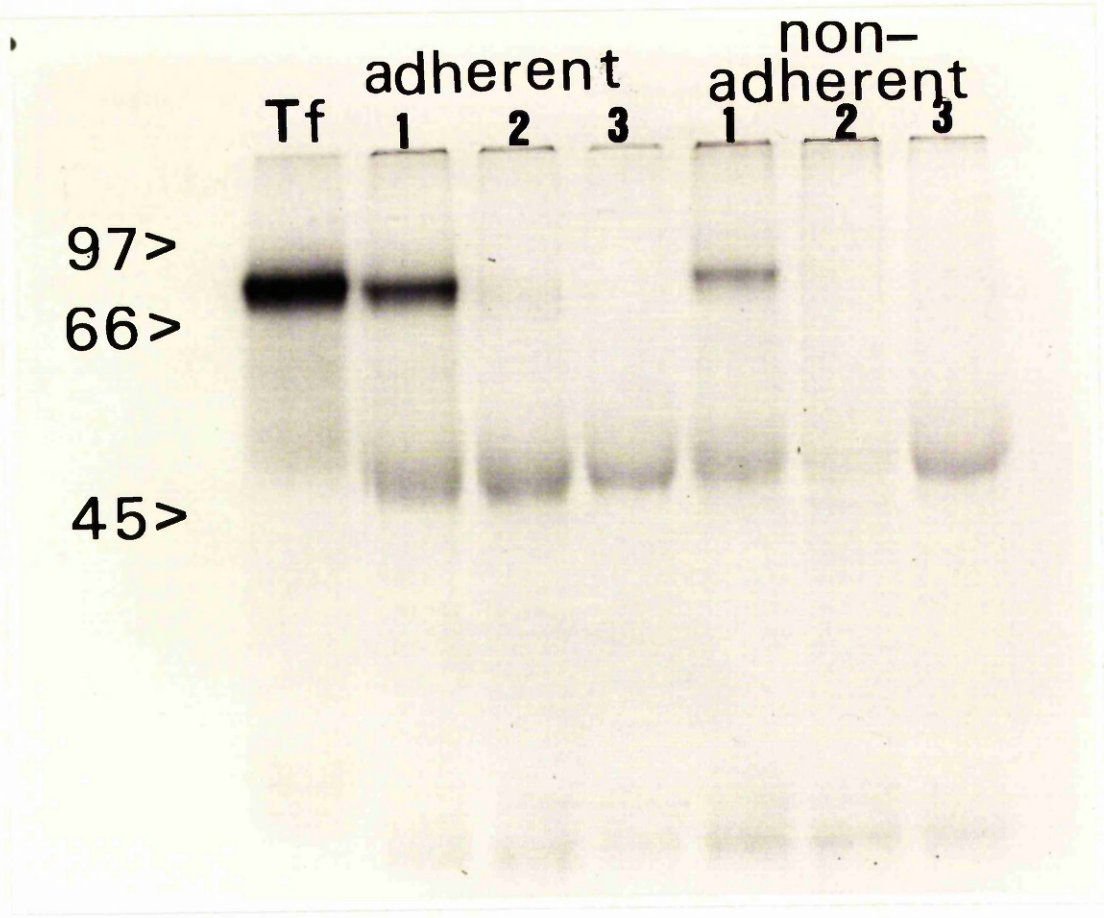

Figure 14 Autoradiography of ^{35}S-cysteine incorporated into transferrin by adherent and non-adherent *in vivo*-stimulated lymph node cells

1: *Test* (+rabbit α-mouse transferrin)
2: *Competition* (+ mouse transferrin, + rabbit α-mouse transferrin)
3: *Control* (+ normal rabbit serum)

immunodepletion step of lymphocytes before performing the assay as described in section 2.3.9. The efficiency of this method was checked by assessing the viability of cells at the end of the incubation period with the antibodies which dropped to around 34%. ANAE stain of depleted cells revealed that the proportion of macrophages rose from 15% in non-depleted preparations to 54% in the depleted cells. **Fig. 15** shows a band corresponding to Tf appears in the test line of the non-depleted lymph node cells. However, lymphocyte depleted cells show a very much stronger band in the test line. Since the same number of cells was assayed in both cases, the less strong band seen in the non-depleted lymph node cells would correspond to Tf synthesised by the macrophages diluted with lymphocytes in the whole lymph node cells. The conclusion is that macrophages are responsible for Tf synthesis upon *in vivo* stimulation of the lymph node.

2.4.6 Intracellular ferritin levels in proliferating mouse lymphocytes cultured with different iron carriers

Turning back to iron incorporation into ferritin which was found to be very low with practically no increase when iron was increased in the medium (section 2.4.4), it was decided to investigate this matter in more detail. Ft synthesis is known to be stimulated by iron in most tissues tested, especially in liver. Therefore, it was of interest to determine the effect of iron on intracellular levels of Ft in lymphocytes, and to investigate its accumulation during Con A stimulation in the presence of different carriers i.e. FeTf, FeNTA, and FePIH at two different iron concentrations (10 and 50 ng/ml) using an IRMA.

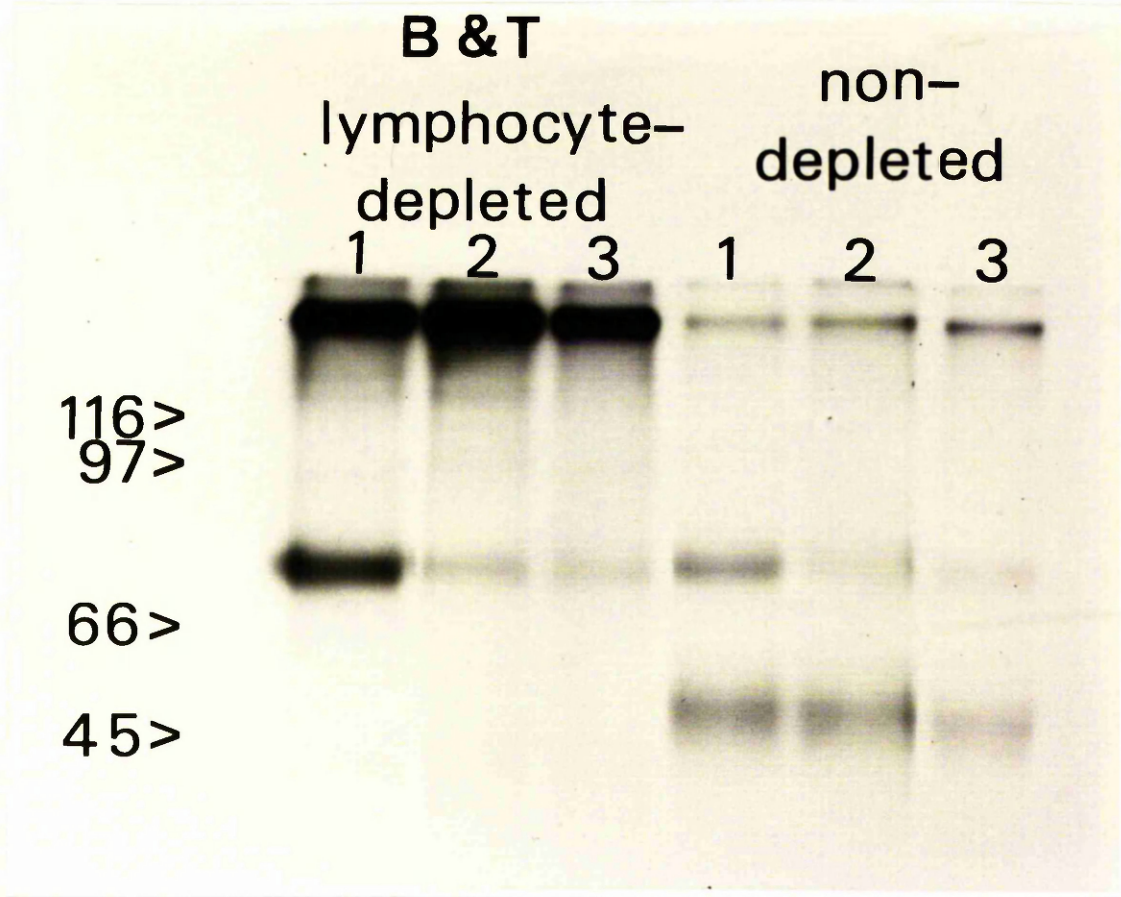

Figure 15 Autoradiography of ^{35}S-cysteine incorporated into transferrin by B and T lymphocyte-depleted and non-depleted *in vivo*-stimulated lymph node cells

1: *Test* (+rabbit α-mouse transferrin)
2: *Competition* (+mouse transferrin, + rabbit α-mouse transferrin)
3: *Control* (+ normal rabbit serum)

The antibody was labelled with the Bolton and Hunter reagent as described in section 2.3.10. The α-MSFt was tested in the IRMA as 1st and 2nd antibody and found to be very weak giving a low dose response curve (**Fig. 16**). On the other hand, the α-MLFt gave a good dose response with liver Ft as standard (**Fig. 17**), hence it was decided to carry out the experiments with only the MLFt-α-MLFt, using a dilution of 1:500 of the antibody.

During the optimization of the method the dilution of the two antibodies was chosen on the basis of finding the lowest working range (**Fig. 17**). The assay did not present the so called hook effect, which can give rise to false low responses at high concentrations of the antigen, up to at least 0.5 μg/ml. The sensitivity of the assay was quite good, as low as 5 ng/ml.

Cellular Ft content was generally low with all iron carriers, but was slightly higher in the presence of Tf, and lower in the presence of FePIH (**Table 2**). At 50 ng/ml iron in the medium, the cells showed only a very modest increase in their intracellular Ft levels compared to cells cultured with 10 ng/ml iron, which indicated that stimulated mouse lymphocytes do not show any marked response to iron by increasing Ft synthesis.

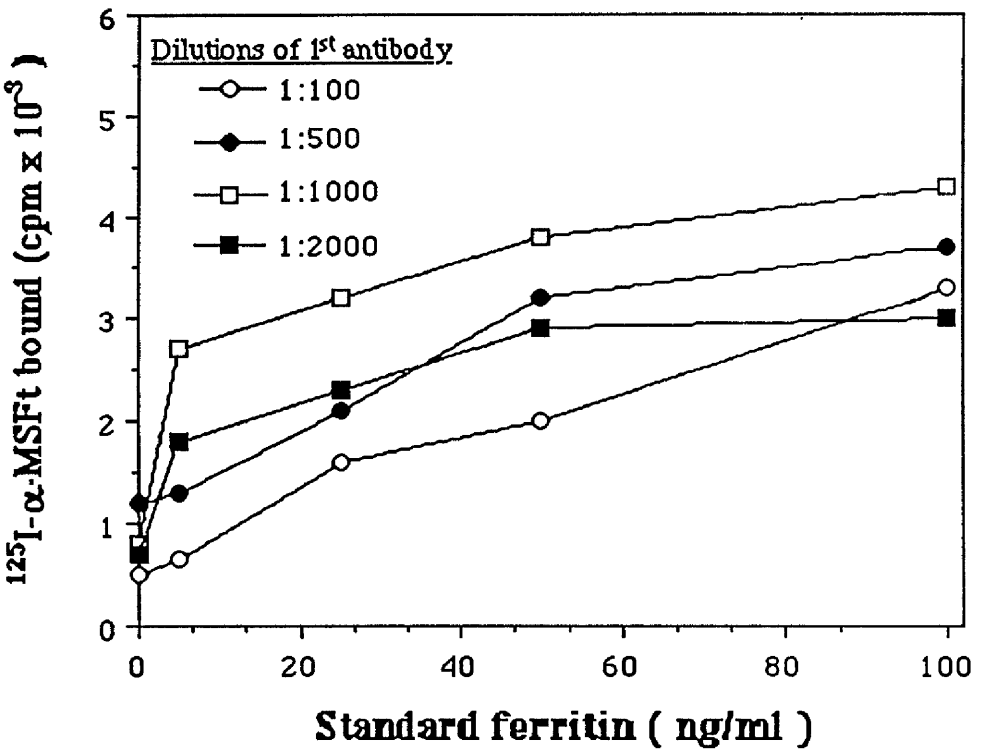

Figure 16 Immunoradiometric assay standard curves with spleen Ft- α-spleen Ft.

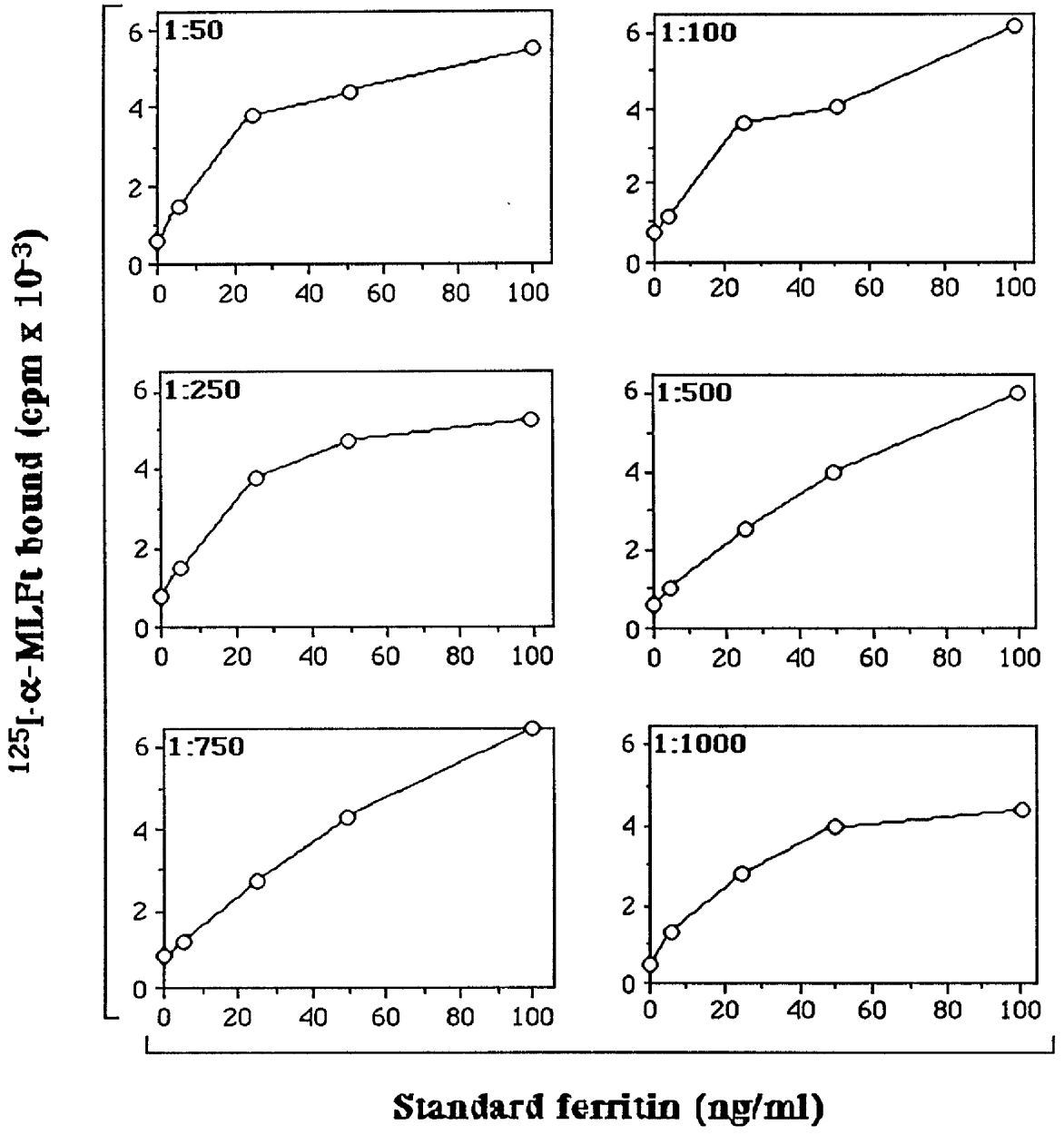

Figure 17 Titration of first antibody (α-mouse liver ferritin) with mouse liver ferritin

Table 2 Intracellular ferritin levels in Con A-stimulated mouse lymphocytes cultured in the presence of different iron carriers

Amount of iron added (ng/ml)	Form of iron	Ferritin content (ng/10^6 cells)
0	—	11.6 ± 3.2*
10 (= 14% Tf sat.)	Fe Tf	16.6 ± 3.0
	FeNTA	14.8 ± 2.5
	FePIH	BD
50 (= 71% Tf sat.)	Fe Tf	24.9 ± 4.8
	FeNTA	19.6 ± 5.0
	FePIH	13.3 ± 2.6

* mean of 4 separate experiments ± SD

BD: Below Detection

2.5 DISCUSSION

Measurement of incorporation of radiolabelled precursors, i.e. ^{3}H-thymidine into DNA was used throughout this study to follow proliferation. Therefore, it was of prime importance to titrate the mitogen. In the absence of Con A the proliferation response was very much lower than when it was present. However, there was still some incorporation of ^{3}H-thymidine by the cells. This might have been the result of non-specific stimulation of some lymphocytes or due to the presence of mesenteric lymph node cells which often contain a proportion of cells that have been antigenically stimulated *in vivo* prior to being cultured. The background stimulation observed was greater when the cells were cultured in serum-containing medium compared to cultures in serum free-medium. Nevertheless, it was always very much lower than the response of mitogen-containing cultures. It was also found that a higher concentration of Con A was needed to induce an optimum proliferation response in serum-containing medium than when serum-free conditions were used. This was probably due to Con A being bound to serum proteins in the medium, causing a reduction in the total number of mitogen molecules available for activation of the cells.

2.5.1 Uptake of iron and thymidine by proliferating mouse lymphocytes cultured in the presence of different transferrin saturations

The uptake of iron by lymphocytes from different iron saturations of Tf showed a gradual increase at Tf saturations below complete

saturation. However, when iron was added beyond the binding capacity of Tf, iron uptake increased dramatically. This suggests that uptake of Tf-bound iron proceeds via the process of Tf-TfR endocytosis, and was controlled via TfR expression, which is modulated by the intracellular iron level rather than the rate of cellular proliferation *per se* (Pelosi-Testa *et al.*, 1988). However, in the presence of excess free iron in the medium uptake rose sharply, suggesting that this uptake took place in a non-specific uncontrolled manner, resulting in an excessive accumulation of the metal inside the cell. The findings of this study agreed well with previous observations concerning other types of cells which showed that cultured Chang cells (Bailey-Wood *et al.*, 1975), liver slices, reticulocytes, and macrophages (Morgan, 1981) took up iron from Tf at rates which depend on the percentage saturation. Below the complete saturation of Tf the iron uptake graphs (**Fig. 4** and **5**) revealed two linear phases. The ratio of iron taken up to the amount of iron present in the medium was constant up to around half saturation of Tf (45%), but when saturation exceeded this level, the proportion of iron uptake to iron available increased. This suggests that diferric Tf donates iron to lymphocytes at a higher rate than monoferric Tf. The initial step in the uptake of iron involves the binding of Tf to specific cell membrane receptors. Transferrin iron exchange has been shown to involve a random loading of iron binding sites, one iron atom at a time (Huebers *et al.*, 1984). On the other hand, the uptake of the FeTf complex by receptors on cell membranes is not random, there being marked preference of receptors for diferric over monoferric Tf. It has been shown in rat hepatocytes (Young and Aisen, 1981) and in rabbit reticulocytes (Young *et al.*, 1984), that the strength of interaction of Tf with the receptor

decreased from diferric to apo-Tf. Diferric Tf was also found to be a better donor for erythroid cells than monoferric Tf (Brown *et al.,* 1975; Hahn *et al.,* 1975; Lane, 1973). However, Verhoef *et al* (1978) have reported that iron uptake by rat bone marrow cells, fibroblasts and lymphoblasts incubated for 3 h was not influenced by the degree of iron saturation of Tf. The reason for this discrepancy could be the short time of incubation used in this study which does not allow uptake to occur. Brock and Rankin (1981) have shown that uptake of iron in mitogen-stimulated lymphocytes is insignificant up to 20 h, and that it occurs mostly between 30 and 70 h incubation. The amount of iron taken up from Tf therefore, depends on the number of each form of the molecule present in the medium, and because of its lower affinity, apo-Tf does not compete significantly for receptors. Thus the degree of transformation of lymphocytes in medium containing partially saturated Tf will most likely depend on the proportion of the different Tf molecular species present. Hence, this study, indicated that at low Tf saturations there are not enough iron loaded Tf molecules and the proliferation was low. An increased rate of transformation is associated with an increased percentage of saturation of Tf with iron, up to the complete saturation of Tf where optimum proliferation occurs. It has been shown that proliferation of mouse lymphocytes was lower in media supplemented with serum from iron-deficient mice than when serum from normal or iron-repleted mice was used (Mainou-Fowler and Brock, 1985). The addition of sufficient iron to bring the iron level of the deficient serum to that of normal serum improved its ability to promote proliferation.

The results of this study suggest that the amount of iron bound to Tf may be important in determining the degree of proliferation. This is in

line with the suggestion that the growth promoting effect of FeTf may be related to the delivery of iron to a specific intracellular site, which is probably related to the iron-requiring enzyme ribonucleotide reductase (Hoffbrand *et al.,* 1976). It seems therefore, logical that with Con A-stimulated mouse lymphocytes, the higher the saturation of Tf with iron the better the *in vitro* proliferation. However the slight enhancement in proliferation seen when lymphocytes were cultured with 'iron-free Tf' compared to control cultures with no addition might suggest that Tf alone may have an enhancing effect. The more likely explanation is that apo-Tf in fact becomes slightly saturated with iron up to 8% (Brock, 1981), due to endogenous iron contamination of the culture medium.

In contrast, the addition of iron beyond the binding capacity of Tf produced the opposite effect on DNA synthesis, i.e. increasing iron levels result in reduction of transformation, indicating that there is no simple correlation between external iron supply and increased DNA synthesis. The presence of Tf might protect the cells from the toxic effects of iron, since non-Tf bound iron added as FeNTA either above saturation of Tf molecules present in the medium or alone was found to be inhibitory to cell transformation. This could be due to iron in this form mediating cytotoxicity as discussed below.

The results obtained in the present study at higher iron saturations of Tf contradict previous findings which demonstrated an optimum effect of Tf around physiological saturation levels (30-70%) with a falling off at higher saturations (Brock, 1981). The discrepancy could lie in a methodological difference between the two systems. In Brock's system, FeTf solutions were made up immediately before use, thus assuming that

completion of iron binding occurred almost instantaneously. However, Zapolski and Princiotto (1977) measured iron binding to Tf by elution from an anion exchange resin column and found that complete iron binding to Tf is not instantaneous, but requires up to 1 h. Therefore, binding of iron to Tf may not have reached completion, and thus, free iron may have existed in these solutions which when added to lymphocyte cultures might have been able to inhibit proliferation as discussed above, even if unsaturated Tf was present in the cultures. It was shown in the present study that iron in the form of FeNTA, which was used to load Tf, is inhibitory to proliferating mouse lymphocytes (see section 2.4.3). Aruoma and Halliwell (1987) reported that iron-loaded Tf (Fe_2Tf) showed no protective ability, but does not itself accelerate $OH^{\cdot}$ production unless chelating agents are present in the reaction mixture, especially if the protein is incorrectly loaded with iron. On the hand in a study reported by Sibille *et al.* (1987) using saturated Tf loaded by a specific procedure (Bates and Schlabach 1973) which was carefully freed of exogenous iron by two cycle of gel filtration they could not find any stimulation of $OH^{\cdot}$ formation by iron loaded Tf at pH 7.4. Moreover, Gutteridge *et al* (1981) showed that Tf was an inhibitor of iron-dependent lipid peroxidation at pH 7.4. All the experiments in this work used FeTf solutions which had been allowed at least 2 h for binding to occur. This difference in experimental technique may, therefore, account for the difference in the results between the two studies when high iron levels were used. Similarly, Broxmeyer *et al* (1983) have reported that iron saturated Tf, but not apoTf decreased the production of granulocyte-macrophage colony stimulating factor (GM-CSF) by PHA- or Con-A-

stimulated human lymphocytes. However these authors achieved Tf saturation by addition of excess of iron salt followed by removal of unbound iron by dialysis and passage through an iron exchange column. It is known that great care has to be taken when iron is added to Tf and it requires the presence of an excess of a low molecular weight chelator, such as citrate or NTA, to prevent hydrolysis, polymerization, and non-specific binding of iron (Bates and Schlabach, 1973). Therefore the inhibitory effect reported by Broxmeyer's group could be due to non-specific iron bound to the Tf molecules becoming detached during the assay and acting as a catalyst for reactions which produce free oxygen radicals.

2.5.2 The effect of different iron chelates on mouse lymphocyte proliferation

Lymphocytes may take up or respond to iron in the medium in two different ways. Firstly they may take up iron physiologically from Tf under the control of cell metabolism and specific receptors on the cell surface. Iron taken up in this form may be used immediately by the cell and thus permit an increase in the cellular metabolic response to mitogens. The other way is uncontrolled non-specific capture of iron and/or transport across the cell surface. The results of this study seem to indicate that uptake of iron from FeNTA is probably an example of the latter. The finding that FeNTA did not promote proliferation suggests that iron bound to NTA was probably handled by cells in a way that failed to make the metal available for metabolic use. It has been reported that free iron leads to formation of polymeric complexes (Spiro *et al.*, 1967). It is possible that this polynuclear iron may bind non-specifically to the

plasma membranes of the cells and exert a toxic effect as will be discussed in the next section. Nevertheless FeNTA, unlike iron salts is known to be quite an efficient chelator in maintaining iron soluble in the extracellular milieu, but intracellularly the iron may polymerise and bind non-specifically to the membrane, as will be discussed in the next section. The finding that as little as 40 ng/ml iron of FeNTA significantly lowered the rate of proliferation to below control levels suggests that the lymphocyte is extremely sensitive to oxidative injury. In comparison, only when the concentration of iron of FeNTA was as high as 10 μg/ml, was the growth rate of RL34 rat liver cells cultured in serum-free conditions affected, while the corresponding of NTA alone gave the same growth as that of the control cells (Yamada *et al.,* 1987). Many *in vivo* and *in vitro* experimental studies exist showing that FeNTA-induced organ injury is related to lipid peroxidation. A single injection of FeNTA was sufficient to produce some hepatic and pancreatic injury and to initiate lipid peroxidation in the liver (Yamanoi *et al.,* 1982). Fodor and Marx (1988) compared ferrous-ammonium sulphate, ferrous-ascorbate, ferric-citrate and ferric-NTA, and showed that FeNTA was by far the most effective in inducing lipid peroxidation of rabbit small intestinal microvillus. The subject of free radicals affecting lymphocytes is discussed more fully later (section 2.5.3)).

The other iron chelate FePIH has been found to support good proliferation of Con A-stimulated mouse lymphocytes. This chelate was identified by Ponka *et al* (1982) and unlike Fe-citrate and FeNTA was found to be able to donate iron to erythroid precursors for use in haem synthesis. In the present study, it was found to be as effective as FeTf

in promoting lymphocyte proliferation at iron concentrations ranging from 40 to 160 ng/ml. These results are in agreements with a report that FePIH permitted growth of embryonic kidney cells (Landschulz *et al.*, 1984) and that the response of these cells was physiological since the cells in the model system used convert to epithelial cells and form tubules. The same group have shown that some other low molecular weight iron chelators such as Fe-acetate, FeNTA, Fe-ascorbate, and Fe-citrate, and unchelated ferrous sulphate could not support proliferation to the same extent as FePIH and FeTf. The present study also confirmed the findings of Brock and Stevenson (1987) with mitogen-stimulated mouse lymphocytes cultured *in vitro* with FePIH in the absence of Tf.

The chemical features of the complex FePIH indicate that it is lipophilic. Landschulz and Ekblom (1985) have found that FePIH has an octanol/saline partition coefficient near 1. This suggests that it can easily and passively enter and leave the phospholipid bilayer of the membrane. The chelator could therefore bypass the TfR and deliver iron directly through the lipid bilayer. Nevertheless, this could not explain why FePIH is more efficient than FeNTA in promoting proliferation since FeNTA was found to donate iron intracellularly (Brock and Rankin, 1981). The finding that levels of iron >160 ng/ml were inhibitory to the cells when bound to PIH suggests that although iron bound to PIH bypasses the TfR route, it is possible that its uptake is less well controlled than that of Tf-bound iron and any excess over that needed for biosynthetic processes becomes toxic for the cells. As will be discussed in the next section, the pattern of intracellular iron distribution suggests that while both chelators bypass the specific uptake mechanism needed to acquire Tf-bound iron, they take different routes once internalized.

The findings that FePIH is equivalent to Tf for supporting proliferation of the cells has some important implications, as it provides another piece of evidence to show that the growth-promoting activity of Tf can be accounted for by its role in providing iron to the cells. Therefore it seems unlikely that Tf could promote cell growth through interaction with its receptor i.e. as a mechanism for transduction of growth signals as has previously been suggested (Brock and Mainou-Fowler, 1983). This conclusion is in agreement with the study of Perez-Infante and Mather (1982) who clearly showed that Tf cannot stimulate growth in the absence of iron, since the effect of apo-Tf on cell growth was blocked by a complete removal of contaminating iron with DFO. Some other iron containing molecules, such as Fe-dextran may also replace the growth promoting effect of Tf on mitogen stimulated lymphocytes (Tanno *et al.*, 1982), as do Hb and haem with 3T3 cells (Young *et al.*, 1979).

2.5.3 Uptake and intracellular handling of iron from different carriers by mouse lymphocytes

The differences in the ability of the two chelators in promoting proliferation compared with Tf, discussed in previous section, suggested that there might be differences in the manner of iron acquisition. To this end, a set of iron uptake experiments were performed using FeTf, FeNTA, and FePIH

The amount of iron taken up from FeNTA was found to be greater

than from FeTf or FePIH, especially at 50 ng/ml iron where a sharp increase in iron uptake was seen. These results are in accordance with the report that mouse lymphocytes took up iron more rapidly from FeNTA and Fe-citrate complexes than from Tf (Brock and Rankin, 1981). Similar findings have been reported with other cell types such as Chang liver cells (White and Jacobs, 1978), which were found to take up 30 times more iron when FeNTA is used as donor compared with FeTf. However, Taylor *et al* (1988) found that iron uptake from ^{59}FeNTA added directly to cultures of mitogen-stimulated peripheral blood lymphocytes was quantitatively very similar to that from ^{59}FeTf. This contrast with the results of the present study is probably due to the fact that their cultures contained 5% serum, the Tf of which could take up ^{59}Fe *in situ* from ^{59}FeNTA.

As discussed in the previous section the explanation of the large difference in iron uptake between FeNTA on one hand and FeTf and FePIH on the other is that iron taken up from the former is not used for metabolic needs, and its accumulation is probably not controlled by any mechanism, whereas iron acquired from FeTf is under control of intracellular metabolism, as will be discussed later. As far as FePIH is concerned, although it can pass through the plasma membrane easily, because of its higher affinity for the metal the chelator would still be able to bind iron intracellularly and donate it to the cell at a slower rate compatible with metabolic needs. Indeed, Taylor *et al* (1988) suggested that although iron utilization is related to cellular activity, the uptake mechanism is only activated when an increase in iron metabolism has exhausted internal stores. However any control of iron uptake from FePIH is achieved by an unknown means other than TfR expression.

Pronase, which inhibits Tf binding to cells probably by digesting TfRs has virtually no effect on the uptake of ^{59}FePIH (Ponka *et al.*, 1979). On the other hand, FeNTA is less likely to be able to traverse the membranes, as mentioned in the previous section. However, Brock and Rankin (1981) have demonstrated that FeNTA donates iron intracellularly to mitogen stimulated lymphocytes and the iron may therefore remain trapped within endocytic vesicles. It could also be that binding of iron to the membranes occurs after entry of the chelator to the cell as will be discussed below.

After determining the differences in radioactive iron delivery to the cell by the different carriers, the subsequent distribution of the metal among intracellular iron compounds was carried out in order to determine the fate of iron taken up, and to interpret their differential effect on proliferation. This was carried out by disruption of the cells and studying the cytosolic phase using affinity chromatography and ultrafiltration.

After its entry to the cell the intracellular pathways of iron are not well defined. After being released into the cell, iron finds its way to multiple specific sites. Since transport of uncomplexed iron is very unlikely, once iron is inside the cell it is presumed to be bound by a carrier. Jacobs (1977) suggested the presence of a labile unidentified low molecular weight iron transit pool in the cytosol which was available for binding by iron chelating agents and which maintained a dynamic equilibrium with many iron-requiring enzymes. Iron chelators could in principle modify iron metabolic pathways by competing with or adding to this cellular iron pool. However, opinions are divided about the

existence of such a pool. The identity of the iron carrier molecules in this fraction are still undefined. There are two possibilities. One is that specific iron-transporting molecules do not exist in the cytosol and that the iron is transported through a variety of iron-chelating amino acids and salts (reviewed by Romslo, 1980). The second possibility is that there is a specific iron-binding and transporting factor with a very high turnover, which makes it very difficult to detect (Bakkeren *et al.*, 1985). Presumably such species are required both within the endosome and in the cytosol. The movement of iron through the labile pool is supposed to be linked to several factors, such as metabolic use of iron for haem synthesis and other iron-containing enzymes (Wrigglesworth and Baum, 1980; Schneider and Erni, 1981). The metabolic state of the cell would dictate the flux of iron from this common pool. Incoming iron would also contribute to the flux into this pool, and it would be affected by every intracellular event involving iron mobilization.

The fraction chelatable by DFO, which probably corresponds to this pool, was found to represent a relatively small proportion in lymphocytes cultured with all carriers tested. These findings are in agreement with the report of Bomford *et al* (1986) that in mitogen-stimulated human lymphocytes, the non-Ft non-haem compartment is initially the largest and the most active for maintenance of cell iron prior to incorporation into different compartments, but gradually this fraction decreased and remained constant after 3 h (only 15% of cellular iron). However, this compartment could also contain iron in non-haem iron-containing enzymes, since in their study they did not further fractionate this compartment. In the present study this low molecular weight fraction

in absolute figures was very much higher in cells cultured with FeNTA at 50 ng/ml iron since these cells take more iron overall compared to cells cultured with FeTf or FePIH. Abnormal expansion of the chelatable pool might be associated with an increased amount of toxic low molecular weight iron which may enhance free radical formation and lead to cell injury as discussed below in the case of cells cultured with FeNTA.

In cells cultured with FeNTA iron was found predominantly in an insoluble non-Ft macromolecular form which probably contains mostly cell membranes, while in cells cultured with FeTf and FePIH, this fraction represents only a small proportion of intracellular iron. This is in line with reports that the high proportion taken up form FeNTA, but not iron originating from Tf was in the pellet of cells probably bound to the cell membrane of mouse lymphocytes (Brock and Rankin, 1981). White and Jacobs (1978) have also shown that iron delivered from FeNTA to Chang cells was found largely in the membrane pellet and very little was in the form of Ft. However, it has been reported that the major part of iron donated by FeNTA to fetal rat hepatocytes was present in the cytosol and not membrane bound as reported with Chang cells (Lescoat *et al.*, 1989), but they suggested that this might be related to iron exchange between FeNTA and Tf resulting in part of the iron entering the cells in a Tf-bound form.

Three hypotheses can be advanced here to explain the nature and origin of the insoluble fraction. Firstly iron in this fraction could simply consist of iron trapped in the membrane. Secondly iron in this fraction could be bound to Hs or Hs-like molecules resulting from saturation and then degradation of Ft to which it was initially bound. Thirdly, iron is

this fraction does not all come classically from Hs, but results from the formation of polymer complexes which then bind to the membranes, especially when iron is taken up non-specifically.

In the case of the first hypothesis, the high proportion of iron in the insoluble fraction in cells cultured with FeNTA could be a result of iron being trapped in the cytoplasmic membrane. Fe^{3+} can only remain soluble at neutral pH by displacing water with ligands of strong chelating agents. The NTA:Fe ratio 5:1 used throughout this study is supposed to be safe and prevent polynuclear complex formation in the medium. However, Marx (1989) has reported that the mononuclear iron form of Fe^{3+} only occurs with an excess of citrate to iron of at least 500:1 and not 20:1 as it was previously thought (Bates and Schlabach, 1975). Therefore some polymers may also be present in the system used in this study when FeNTA was used. Electron microscopic evidence for the presence of electron-dense materials on the surface of lymphocytes incubated in the presence of Fe-citrate have been presented (Nishiya *et al.*, 1980). Landschulz and Ekblom (1985) showed that FeNTA has a low partition coefficient between octanol and saline (<0.01), implying that iron associated with the cells probably consists of polynuclear iron bound non-specifically to the cell, and they concluded that FeNTA cannot traverse the lipid bilayer. However, White and Jacobs (1978) have shown that iron in the membrane pellet of Chang cells cultured with FeNTA was not removed by DFO, which suggests that the binding of iron to the membrane occurs after entry into the cell rather than extracellularly, assuming it is bound to the membrane. This finding does not support the hypothesis that iron uptake from FeNTA occurs by adsorption of the chelate on the cell surface rather than intracellularly.

With regard to the second hypothesis, it is known that lymphocytes are not specialised for iron storage the potential capacity of these cells to synthesise Ft to sequester iron in a harmless form is not high (Summers *et al.*, 1975; Lema and Sarcione, 1981; Summers and Jacobs, 1976; Dörner *et al.*, 1980; Pattanapanyasat, 1988; 1989b, and confirmed in this study, see section 2.4.6). As a consequence, the large amount of iron taken up from FeNTA would be quickly transported from the labile iron pool and load the limited amount of Ft present, which would result in rapid Ft degradation and the generation of Hs. It is known that the excessive loading of Ft is followed by lysosomal uptake of the molecules and their subsequent degradation (Trump *et al.*, 1973; Weir *et al.*, 1985), which is generally considered to be a relatively inactive form of storage iron (O'Connell *et al.*, 1986a, 1986b). The polymerization of Ft molecules in solution has been recognized for some time, and Niitsu and Listowsky (1973) have reported that this process preferentially affects iron-rich molecules, with the incorporation of rich molecules into lysosomes, degradation of the protein shell, and formation of Hs. Indeed, It has been shown that while Ft is the major iron protein present in the normal liver, in iron overload Hs predominates (Selden *et al.*, 1980). Therefore, it might be assumed that in the present experiments, this process is accelerated when cells are incubated with FeNTA due to the high level of uptake. However even when the concentration of FeNTA in the medium is low (10 ng/ml) and the subsequent iron uptake comparable to that from Tf at 50 ng/ml, the abnormal distribution between cytosol and membrane iron remains with a larger accumulation in the latter. Ultrastructural studies of liver in artificially iron-loaded animals (Trump *et al.*, 1973) or in human liver

biopsy material (Iancu *et al.*, 1977) suggest that iron loading results in the accumulation of Ft in lysosomes at which sites it is converted to Hs. This is in agreement with other studies with liver and heart cells (Iancu *et al.*, 1987; Jacobs *et al.*, 1978; Link *et al.*, 1985) which found that unlike the physiological uptake of Tf iron which is primarily located to cytosol, low molecular weight iron complexes, such as FeNTA and Fe-ammonium citrate are rapidly confined to siderosomes. This hypothesis could be valid especially in cases of physiological uptake of the metal which implies that excess internalized iron would pass through Ft before the former is converted to Hs. However, in cases of non-physiological uptake it could be that Hs is not the only insoluble iron storage compound and polymers of iron complexes might also exist.

Turning to the third hypothesis, it is possible that once inside the cell, FeNTA might result in abnormal intracellular iron processing due to dissociation of iron from the carrier and release of large quantities of free iron. This could saturate all available natural low molecular mass compounds capable of binding iron inside the cytosol. This iron could accumulate, polymerise and precipitate, leading to subsequent non-specific lysosomal incorporation of some of these polymers, adding to the to iron in Hs derived from Ft. Therefore the insoluble fraction of these cells would in fact consist of both free and lysosomal-bound iron polymers together with Hs. Although there was no evidence that polymerization occurs in the cytosol, and FeNTA is thought to be an excellent chelator in maintaining extracellular iron in a soluble form, this might occur if the chelate was subject to degradation by the metabolism of the cells. The hypothesis that FeNTA could be subject to degradation by

the metabolism of the cell is supported by the findings of Nakamoto *et al* (1986) who incubated Ehrlich ascites tumour cells with ^{59}FeNTA and Fe-nitrilotri(^{14}C)acetate at NTA:Fe of 5:1 and found that the intracellular ratio NTA:Fe decreased as the extracellular concentration of FeNTA increased reaching almost 1:1 at an iron concentration of 3.6 mM.

Thus the conclusion is that the way the cells handle iron may depend on the form in which it was supplied. If the second hypothesis is valid in the case of cells cultured with FeTf, the third one is more likely to be true especially in the case of cells cultured with FeNTA, although the first hypothesis can not to be excluded in this case.

If FeNTA binds non-specifically to the membrane and polynuclear iron complexes were unable to cross the membrane as discussed above they may exert their toxic effect at this site by providing active iron which may participate in hydroxyl radical generation which promotes lipid peroxidation, leading to cell damage. This view is in accordance with the report of Soyano *et al* (1985) that the inhibitory effect of FeNTA on lymphocyte proliferation is due to formation of iron polymers which affect the membrane of these cells. Landschulz and Ekblom (1985) have also reported that the toxic effect of FeNTA on kidney tubules is due to alteration of their membranes by polynuclear iron.

If FeNTA was able to enter the cell as discussed above, is it iron that is incorporated into Hs after Ft degradation following excessive uptake into the cell that is responsible for the inhibitory effect on lymphocyte proliferation, or is it formation of polymeric iron after degradation of the chelator and liberation of iron inside the cell? Alternatively, toxicity might be a function of the abnormally large

intermediate pool which in absolute figures is very much greater when cells were cultured with FeNTA at 50 ng/ml.

When cells were exposed to a lower iron concentration (10 ng/ml) in the form of FeNTA, toxicity did not occur (as judged by ^{3}H-thymidine uptake which was similar to control cultures) and the cells were probably able to sequester iron in Ft, with formation of Hs which also represented the major fraction of cellular iron of these cells. However when cells were cultured with 50 ng/ml iron, this fraction represented the same proportion as when cells were cultured with 10 ng/ml and yet proliferation was reduced as compared with control, suggesting that possibly there is polymeric iron in this fraction. If this assumption is true most of iron in this fraction in cells cultured with 10 ng/ml iron would probably be in Hs and very little or none in the form of polymers. A close positive correlation has been shown between enhanced lysosomal fragility and liver Hs content (Selden *et al.,* 1980). Hs accumulation within tissues could provoke lysosomal damage by increasing lipid peroxidation (Selden *et al.,* 1980). However this does not necessarily imply that Hs is the cause of cytotoxicity. On a unit iron basis, Hs has been found to promote $OH^{\cdot}$ generation to a much smaller extent than Ft, which suggests that iron in the form of Hs is far less active in promoting lipid peroxidation (O'Connell *et al.,* 1985). Moreover, addition of Hs to normal liver homogenates is incapable of promoting lipid peroxidation (Bacon *et al.,* 1985). It could thus be argued that conversion of Ft into Hs is biologically advantageous, in that it would diminish the occurrence of oxygen-radical reactions in the presence of excess iron in the medium. It is therefore likely to be that the iron deposits in the cell represent

immobilized metabolically inactive iron. Hence Hs formation appears to be a protective mechanism and overload of the intermediate pool may be responsible for toxic deposition of iron.

In conclusion, rather than being an instrument of cellular damage, the formation of Hs in response to iron loading may initially represent an important mechanism protecting cellular organelles against iron toxicity. However, with increasing iron uptake this protective mechanism may no longer be sufficient and polynuclear iron formed after degradation of FeNTA following abnormal saturation of low molecular iron complexes in the intermediate pool may be responsible for its participation in peroxidation damage.

What makes FePIH better than FeNTA in this respect? PIH has a very high and specific affinity to Fe^{3+} especially at neutral pH (Richardson *et al.,* 1989) Its chelating efficiency on a weight-per-weight basis is equal to or slightly better than DFO (Hershko and Weatherall, 1988), and since the latter is known to inhibit iron-dependent $OH^{\cdot}$ production (Gutteridge *et al.,* 1979; Halliwell, 1985), at least under certain conditions (Braughler *et al.,* 1988), this would suggest that FePIH must be very efficient in preventing the reduction of Fe^{3+}, and thus preventing it participating in radical formation. This view is supported by the report of Mello Filho *et al* (1984) which showed that 2,2'-bipyridine, an iron chelator related to PIH, prevents both DNA damage and the killing of mouse cells by H_2O_2 by entering the cells and chelating their intracellular iron. However, bipyridine is an Fe^{2+} chelator and the mechanism of action might be different.

The results of size of the intermediate molecular weight fraction fit

very well with the results of thymidine uptake. This fraction, which represents iron incorporated into non-Ft non-Tf iron-containing proteins and thus reflects the metabolic activity of the cell, was found to represent relatively the largest proportion in cells cultured with FeTf or FePIH. Iron in this fraction is bound to many enzymes and coenzymes. Probably the most important of all as far as these cells are concerned is ribonucleotide reductase (Brown *et al.,* 1969). The importance of this enzyme in DNA synthesis was shown by Brockman *et al* (1971) who reported impaired incorporation of ^{3}H-thymidine into DNA by a leukaemic cell line when the cells were cultured in medium containing inhibitors of the ribonucleotide reductase system. However, in cells cultured with FeNTA this fraction represents a smaller proportion.

The finding that the fractionation profile of iron acquired from FePIH is similar to that of iron acquired from FeTf indicates that the former complex might be capable of donating its iron to the intermediate low mass compound(s), in which iron is fed *en route* to functional iron-containing enzymes or the storage compound Ft, at equal rate to FeTf. Alternatively, FePIH might simply jump this step and the actual complex fulfills this role. The last probability is more likely to be true since FePIH can pass directly to the cytosol (Landschulz and Ekblom, 1985) and its ability to bind iron more strongly than FeNTA would enable it to deliver its load to the mitochondria to join the normal metabolic pathways. This might also reflect the fact that one part of the PIH molecule, pyridoxal, a form of vitamin B_6, is a natural biological substance (Ponka *et al.,* 1979). This same argument can be applied to explain the means by which FePIH donates iron to the cell, in which case

one cannot exclude the possibility that PIH crosses biological membranes using a transport system for pyridoxine derivatives. However, Solomon (1982) demonstrated that pyridoxal uptake by red cells was not affected by ATP depletion or addition of inhibitors of anion transport channels. This seems to indicate that red cell uptake of pyridoxal is not dependent upon a specific transport mechanism and both pyridoxal and PIH may just diffuse non-specifically across the cell membrane. On the other hand, Ponka *et al* (1979) have reported that ATP seems to be required for iron mobilization from reticulocytes by PIH, suggesting that the whole chelate unlike its pyridoxal moiety, may be taken by an active transport system.

It is worth noting that with all carriers the overall intracellular distribution of iron among different intracellular compartment was not altered by changes in the amount of iron available. These results are in agreement with the findings of White and Jacobs (1978) with Chang cells.

In previous reports using cultured fibroblasts (Octave *et al.*, 1981), human erythroleukaemic cells (Klausner *et al.*, 1983), or established liver cell lines such as Chang cells (White *et al.*, 1976), the majority of iron taken up from Tf was sequestered in Ft. In sharp contrast, the Ft-iron fraction was by far the lowest in all cultures of lymphocytes. However at both concentrations tested it was slightly larger in cells cultured with FeTf. Under increased metabolic demands for iron the major source of iron to be used would be that present in the low molecular weight fraction. In view of these results, which suggest a very limited ability of these cells to incorporate iron into Ft, it was of interest to investigate Ft accumulation in response to iron presented in different forms.

The IRMA for mouse Ft used in this study to measure the lymphocyte content of Ft detected predominantly the L type, since the antibody used was an α-mouse liver Ft. Although there was a relative difference in the level of Ft in proliferating mouse lymphocytes cultured with three different iron carriers when the iron concentration in the medium was increased, this difference was not very pronounced in comparison to other cell types such as monocytes (Dörner *et al.*, 1983b). This suggests that there is a basal level of Ft which could not be greatly increased by the addition of any form of iron to the incubation medium. However, Jones *et al* (1983) have reported that normal human peripheral blood lymphocytes contain a relatively high proportion of H subunits, and incubation of these cells with iron increases preferentially the H subunits (Worwood *et al.*, 1984). This could partly explain the relatively low Ft content detected in these cells in the present study. A low increase in L-rich Ft in response to iron has also been reported by other authors (Pattanapanyasat *et al.*, 1988; Pattanapanyasat, 1989a). However, the control of Ft synthesis is much less well studied in mouse lymphocytes than in human lymphocytes, and it is not really known if mouse Ft behaves similarly. Nevertheless, since the cells in the present work were incubated for a relatively long period its was more relevant to assess the L-rich ferritin which unlike H-rich Ft is more stable and better suited for long-term iron storage (Drysdale, 1988). Together with the proliferative activity of the cells this can give a relative idea of how these cells are succeeding as far as iron detoxification is concerned. The results of the present study are in agreement with some reports which showed that addition of either iron (Summers *et al.*, 1975) or iron saturated Tf (Summers and Jacobs, 1976), failed to induce human

lymphocyte Ft synthesis *in vitro*. These results are also in accordance with the findings of Pattanapanyasat *et al* (1988) which showed that there was a significant increase in Ft synthesis in lymphocytes cultured in the presence of PHA when compared to non-stimulated cells, and that the presence of FeNTA made little difference to the rate of Ft synthesis. Lema and Sarcione (1981) have reported that administration of increasing amounts of iron to rats *in vivo* induced both liver and peripheral blood lymphocyte Ft synthesis, but incubation of liver and lymphocytes with increasing concentrations of iron *in vitro* stimulated liver but not lymphocyte Ft synthesis. These data suggest that induction of lymphocyte Ft synthesis observed after iron administration *in vivo* resulted from secondary stimulatory mechanisms rather than to iron *per se*. The results of the intracellular Ft content together with the results of the intracellular distribution of iron lend support to the theory of Mattia *et al* (1986) who proposed that the regulation of intracellular iron distribution is dependent upon absolute Ft levels and is unaffected over a range of amounts of iron delivered to the cells

The fact that proliferating mouse lymphocytes cultured with both physiological and non-physiological forms of iron showed no marked increase in synthesis of Ft could be interpreted as indicative that these cells are directing most of their iron either to a metabolically inert pool in the case of FeNTA (which failed to support proliferation) or into enzymatic needs (in the case of cells cultured with FeTf and FePIH) as judged by the intracellular distribution of iron (see section 2.4.4). In cells specialized for iron storage where the potential accumulation of the metal is high, iron can regulate the synthesis of Ft by translational

control mechanisms. It has been generally considered that the main function of Ft in these cells is to store iron not immediately required for metabolic activity and release it to metabolic iron pools when required. It is well known also that most cells and tissues, when loaded with iron, incorporate the metal into Ft. Increased synthesis also occurred during inflammation and during cell differentiation (Fibach *et al.*, 1985). Since the main function of Ft in lymphocytes is storage of iron for intracellular use (house keeping) rather than for use by other cells the mechanisms of regulation could differ.

2.5.4 Transferrin synthesis by mouse lymph node cells

It is well established that the major site of synthesis of Tf is the liver (Morgan, 1981). Nevertheless, extrahepatic sites of Tf synthesis may exist (section 1.1.2.1). In the course of the present study the initial suggestion of Tf synthesis by mouse lymph node cells came when Tf was detected after chromatographing the lysate of cells cultured with ^{59}Fe-labelled chelator in the absence of any exogenous Tf. Early studies reported that the human lymph node (Prunier *et al.*, 1964) makes Tf, and it has also been reported that human lymphocytes might have the capacity to secrete Tf (Soltys and Brody, 1970; Nishiya *et al.*, 1980; Broxmeyer *et al.*, 1983). This was confirmed by the study of Lum *et al.*(1986) who showed that activated human helper T-lymphocytes synthesise Tf. Therefore, to investigate whether this is also true for mouse lymphocytes a study of Tf synthesis by those cells was carried out.

In this study the mouse lymph node was found to be one of the extrahepatic local sites of Tf synthesis. Tf was found to be actively synthesised by lymph node cells upon *in vivo* immunological stimulation but not by quiescent cells. However, the present study has failed to demonstrate the synthesis of the other smaller Tf-like molecule (60 Kd) which was found to be synthesised by activated human lymphocytes (Lum *et al.,* 1986). Moreover *in vitro* Con A-stimulated cells failed also to show an active synthesis of the protein. All this might suggest that another type of cell could be responsible for Tf synthesis within the lymph node. Therefore, it was of interest to determine which particular cell populations within the tissue are involved.

It has been known for some time that macrophages can synthesise Tf (Phillips and Thorbecke, 1966; Haurani *et al,* 1973). It has also been suggested that endogenous Tf might act as a vehicle for the release of iron from macrophages (Haurani and Ballas, 1984). These cells, which are in close contact with lymphocytes within the node, were found to increase sharply after *in vivo* stimulation of the node. All this makes the macrophage the most likely candidate. In subsequent experiments it was confirmed that these cells are responsible for Tf synthesis in the stimulated lymph node. As mentioned earlier, the initial detection of newly synthesised Tf was in lysates of Con A stimulated lymph node cells cultured in Tf-free medium and was possible because of its content of radioactive iron. The reason for the failure of the immunoprecipitation method to detect Tf synthesis in these cells might be explained by the possibility that the detection by the radioactive iron content of the protein is more sensitive than the autoradiography of ^{125}I-labelled Tf. But

whether this iron was incorporated by Tf molecules endogenously or occurs after lysing the cells is not known.

The mechanism that regulates the expression of the Tf gene in the lymph node and the physiological role of Tf during the immunological activation process are presently unknown. It is possible that the Tf gene in these cells is not expressed in the resting stage under normal conditions, and that in the host immune response the gene is switched on to provide a source of available iron in an autocrine/paracrine manner to support localized proliferation of those rapidly dividing cells. This is in agreement with finding of Vostrejs *et al* (1988) who reported that Tf synthesis in a lung cancer cell line increased more than 10-fold when cells entered active phases of the cell cycle, a time when Tf and particularly iron is necessary for cell division to proceed.

The need for a local supply of Tf to these cells may result from the restricted environment within the lymph node during immunological stimulation, when iron is being withheld from circulation (Weinberg, 1984). During acute phase reaction such as bacterial infections or neoplasia the body responds with a protective reduction in the concentration of available iron resulting in a rapid drop in the iron saturation of circulating Tf (Zarrabi *et al.*, 1977; Lee, 1982; Finkelstein *et al.*, 1983), by mechanism(s) which are not fully defined yet. Moreover, in both aseptic and microbial inflammation Tf concentration is reduced (Kumar *et al.*, 1978). This is assumed to be a characteristic of the host defence mechanism against microbial pathogens and neoplasia (Ballantyne, 1984; Hunter *et al.*,1984), since under these conditions serum iron is less accessible to the pathogen. On the

other hand, under these conditions, shortages in iron supply could affect the proper function of the immune cells which are liable to find themselves in a state of suddenly increased activity caused for example by invasion of foreign organisms. This is especially true within lymphoid organs where a sudden increase in demand for iron may not be met fast enough resulting in impaired immune response. Cells of the lymph node would then be better equipped if they could acquire iron independently of plasma Tf by synthesising their own Tf locally in order to proliferate and exert their effector functions. Therefore, macrophages may serve as an intermediary in the transport of iron to proliferating lymphocytes under certain conditions. Specialized cellular proliferation *in vivo* by tissues that are not well vascularized might be limited by insufficient delivery of Tf-bound iron from plasma. A striking example of these tissues which is quite similar in certain aspects to the lymph node, is the testis in which Tf synthesis by Sertoli cells provides iron to proliferating spermatocytes (Skinner *et al.*, 1980). The brain, which is separated from blood stream by the so-called blood-brain barrier, has been shown to synthesise Tf (Levin *et al.*, 1984; Aldred *et al.*, 1987), which contributes to maintaining homeostasis in the extracellular environment of the brain.

Thus lymphocytes may be capable of modulating their own supply of iron through influencing the regulation of macrophage Tf synthesis via lymphocyte-macrophage contacts and/or diffusible substances. By analogy to this, Le Magueresse *et al* (1988) have found that removal of germ cells contaminating the Sertoli cell cultures resulted in a significant decrease in Tf secretion. This hypothesis awaits further investigation.

CHAPTER THREE

THE EFFECT OF DIFFERENT LEVELS AND FORMS OF IRON ON HUMAN LYMPHOCYTES

3.1 INTRODUCTION

The preceding chapter reported evidence for the importance of iron availability on the proliferation of mouse lymphocytes in response to mitogens and that iron in the form of FeNTA, but not Tf-or PIH-bound iron had an inhibitory effect. Iron bound to NTA was probably toxic to the cells. Although the use of cells from an animal model has allowed variables which could complicate the interpretation of the results such as infections, age and nutritional differences to be eliminated, it was of prime importance to perform comparable studies on human lymphocytes. If the inhibitory effect of iron under the conditions reported in the previous chapter with mouse cells is applicable to human lymphocytes, the immunological status of humans may be seriously impaired under similar conditions. It is thus intrinsically more valuable to study human lymphocytes which would also enable comparison to be made with clinical situations. Therefore one of the aims of the work presented in the present chapter is to determine to what extent the findings with the mouse cells apply to human cells, and to investigate whether the way cells from these two species behave in the presence of different levels and forms of iron is different. It is known that these cells are exposed to different conditions *in vivo*, as Tf saturation in the plasma of mice is higher that in humans. *In vivo* saturation of mouse Tf with iron is 65-80% (Puschmann and Ganzoni, 1977; Kuvibidila *et al.*, 1983) against about 30% in humans (Morgan, 1981). Thus the former may be expected to be better equipped than the latter in coping with excess iron. It could therefore be hypothesized that the inhibitory effect of excess iron would be more

pronounced in the human system. The work reported in the present chapter is therefore an extension of these studies to normal human lymphocytes, which examines the effect of different forms of iron on lymphoblastic transformation.

The results reported in the previous chapter also gave some evidence that mouse lymphocytes do not have a marked capacity to synthesise Ft in response to iron in the medium. To investigate whether the same is true in human lymphocytes, Ft synthesis in proliferating normal human lymphocytes has been investigated in more detail, by looking at the effect of a larger range of iron concentrations on cellular Ft content. The results reported with mouse cells suggested that lymphocytes were unable to proliferate because they could not efficiently handle excess iron by increasing Ft synthesis as discussed above (section 2.5.3).

Several studies have suggested that besides its well known antimicrobial activity, Lf posses several characteristics which could implicate it as potential modulator of immune cell function (Duncan and McArthur, 1981; Brock, 1985). In this respect, the release of this protein and its local accumulation in inflammation are of particular relevance. Therefore Lf might have a role in controlling lymphocyte proliferation. It was also hypothesized that Lf could sequester excess iron in the medium and could prevent its toxic effect on the cells, and hence enable the cells to proliferate when excess iron is present. However no definite proof for this is available. Therefore, in this study Lf was tested for its effect on human lymphocyte proliferative response and in particular to address the question of whether this protein which has

a much higher affinity constant for iron than Tf (Aisen and Leibman, 1972) is capable of overcoming the inhibitory effect of excess iron when all the Tf present in the medium is saturated. The study was performed on human rather than mouse cells because it is quite easy to prepare sufficient quantities of human Lf from colostrum, whereas mouse Lf is not readily available.

In the previous sections (2.4.2 and 2.4.3) it was shown that iron modulates proliferation of T-cells. These cells consist of different subsets exerting different functions in the process of an immune response. The results reported in the previous chapter of a poor *in vitro* response of lymphocytes to mitogen stimulation in the presence non Tf-bound iron in the form of FeNTA suggests that impairment may occur to the clonal expansion of these cells which is in line with previous reports (Good *et al.*, 1986; 1987). Depression of the specific immune response in iron overload is less well documented than in iron deficiency, although disturbances have been reported in iron overload-related diseases (reviewed by de Sousa, 1989). However correlation between T-lymphocyte subset abnormalities and iron loading remains quite speculative. There is little information on whether different iron conditions can affect the ratio of lymphocyte subsets *in vitro*. Therefore, to address this question and to see whether the inhibitory effect on proliferation is ascribed to action on all T-cell subsets irrespective of surface phenotype, experiments investigating the effect of different concentrations of iron bound to chelates or Tf were performed to investigate which subsets are altered by iron. This would further our understanding of the immunological.regulatory properties of iron.

It is well established that transformed cells generally require less serum growth factors than normal cells (Draser and Irving, 1973). A non-Tf-mediated pathway of iron incorporation has been demonstrated in malignant cell-derived lines in culture (Taetle *et al.*, 1985; Basset *et al.*, 1986), suggesting that neoplastic cells might have different mechanisms for regulating their requirements of the metal. Therefore the study performed on normal human lymphocytes was extended to a human leukaemic cell line CCRF-CEM. This was carried out to investigate whether these cells, which have the property of unremitting proliferation, respond in the same way to iron as their normal homologue undergoing mitogen-stimulation, which leads to terminal differentiation. In addition, Tf synthesis in CCRF-CEM cells was investigated to see whether, as in the activated normal human T-lymphocytes (Lum *et al.*, 1986), the Tf gene is expressed in these cells. This could serve as an extra means of acquisition of the iron that is needed by these constantly transformed cells. In this respect, endogenous cellular production of Tf could serve in an autocrine growth regulatory capacity, and uncontrolled Tf biosynthesis by these neoplastic cells could be an important mechanism by which a neoplasm might establish autonomous proliferation, and some evidence for this has already been presented (Kitada and Hays, 1985; Morrone *et al.*, 1988).

In summary therefore, the work reported in this chapter aims to investigate:

1) The effect of different forms and amounts of iron on transformation of human lymphocytes and the T-cell line CCRF-CEM and compare them with the results obtained with mouse

cells.

2) The effect of iron on cellular Ft levels in human lymphocytes in relation to its effect on proliferation.

3) The effect of the Lf on lymphocyte proliferation.

4) The effect of different forms and amounts of iron on the expression of T-cell subset markers.

5) Tf synthesis by CCRF-CEM cells.

3.2 METHODS

3.2.1 Cell culture conditions of human mononuclear cells

Peripheral blood was obtained from healthy volunteers aged 19-48, collected into sterile plastic universals containing 50 IU/ml heparin (Leo laboratories Ltd, Princes Risborough, Bucks, UK) and mixed 1:1 with RPMI-1640 medium. Mononuclear cells were isolated by density gradient centrifugation through lymphocyte separation medium (Flow). The cells were washed twice with RPMI-1640 medium, viability assessed using eosin exclusion, and finally resuspended in complete medium composed of RPMI-1640 supplemented with penicillin-streptomycin at 100 IU/ml and 100 μg/ml respectively, 0.3 mg/ml L-glutamine, and HSA at 1 mg/ml. The cells were cultured at a concentration of 2×10^6 cells/ml in plastic conical test tubes in the presence of purified phytohaemagglutinin (Wellcome, Dartford, England) at appropriate concentrations. The cells were cultured at 37° C in an atmosphere of 5% CO_2, 95% air for 72 h. Proliferation responses were assayed as described in section 2.3.2 with mouse cells.

3.2.2 Indirect immunofluorescence

After terminating the incubation period, cells which had been cultured as above were washed twice with PBS/FCS (5%). Three tubes containing aliquots of 5×10^5 cells each were put on ice. The cells were

spun at 800 rpm for 5 min at 4° C, the supernatant aspirated, and cell pellets were left with as little liquid as possible. To each tube was added 10 µl of a 1:20 dilution in PBS of one of the following monoclonal antibodies; α-CD3, α-CD4, and α-CD8 (Serotec). The tubes were well mixed on a vortex, and left incubating for 20 min on ice. During the incubation period each tube was vortexed twice. Tubes were then centrifuged and cells were washed three times in PBS/FCS to remove any unbound antibody. Cell pellets were left with as little liquid as possible and 20 µl of a 1:32 diluted fluorescein isothiocyanate (FITC) conjugated F(ab')2 fraction rabbit-α-mouse immunoglobulin (Serotec) was added to the cells which were again incubated on ice for 20 min. Following the second incubation period, the cells were washed as above and diluted to 0.7 ml with PBS/FCS. Cytopreparations were prepared by spinning 100 µl of each sample on to a microscope slide at 800 rpm for 5 min using a Shandon-Elliot cytocentrifuge. The slides were taken out, allowed to air dry and then fixed with ethanol/glacial acetic acid (95%/5%) for 20 min at -20° C. Following cell fixation, slides were washed by transferring them into 3-4 changes of fresh PBS. The slides were then mounted in 90% glycerol/10% PBS containing 0.1% sodium azide under a coverslip and sealed with nail varnish. The cells were observed under oil immersion in an Olympus BH-2 fluorescent microscope, which allows the observation of cells by both ultraviolet and phase contrast. This enables the fluorescent cells to be counted under UV light and the total mononuclear cells under phase contrast. Four random fields were chosen, and 200-280 cells were counted per slide.

3.2.3 Preparation of lactoferrin

The isolation of this protein was performed following the method reported by Johansson (1969), with some variations. Human colostrum (30 ml) (kindly provided by staff of the breast milk bank, Yorkhill Hospital, Glasgow, Scotland) was spun at 1250 *g* for 30 min at 4° C. Fat and debris were discarded, and the aqueous layer was collected. The defatted colostrum was diluted with 4 volumes of sterile saline. FeNTA (10 mM stock solution) was added (30 µl/ml of original volume of colostrum) which turned the colour of the colostrum solution to red-pink. Dry CM-Sephadex C50 (Pharmacia) (0.5 g) was added and the mixture gently stirred for 1 h. After allowing the gel, which turned to a reddish colour, to settle, the supernatant was aspirated, and the gel was washed with 3 changes of 50 ml of saline with 5 min gentle stirring each time. The gel was then packed into a small column made from a 20 ml syringe barrel with a Millipore prefilter in the bottom. To elute contaminating proteins a reservoir of 0.05 M Tris HCl, pH 8.0, was connected and allowed to run through until no further protein eluted, as judged by reading the optical density at 280 nm. The reservoir buffer was changed to 0.05 M Tris/2.0 M NaCl, pH 8.0, to elute the Lf and fractions of 1.5 ml were collected. The optical densities at 280 and 470 nm were measured (**Fig. 18**), and the Lf peak collected. The figure shows that there was a perfect match between the protein peak fractions and the red colour indicative of their iron content, demonstrating a good separation procedure. The fractions comprising the main peak were collected and the deep red solution was then concentrated by ultrafiltration and dialysed against 4 daily changes of PBS, pH 7.2 at 4° C. The solution was then

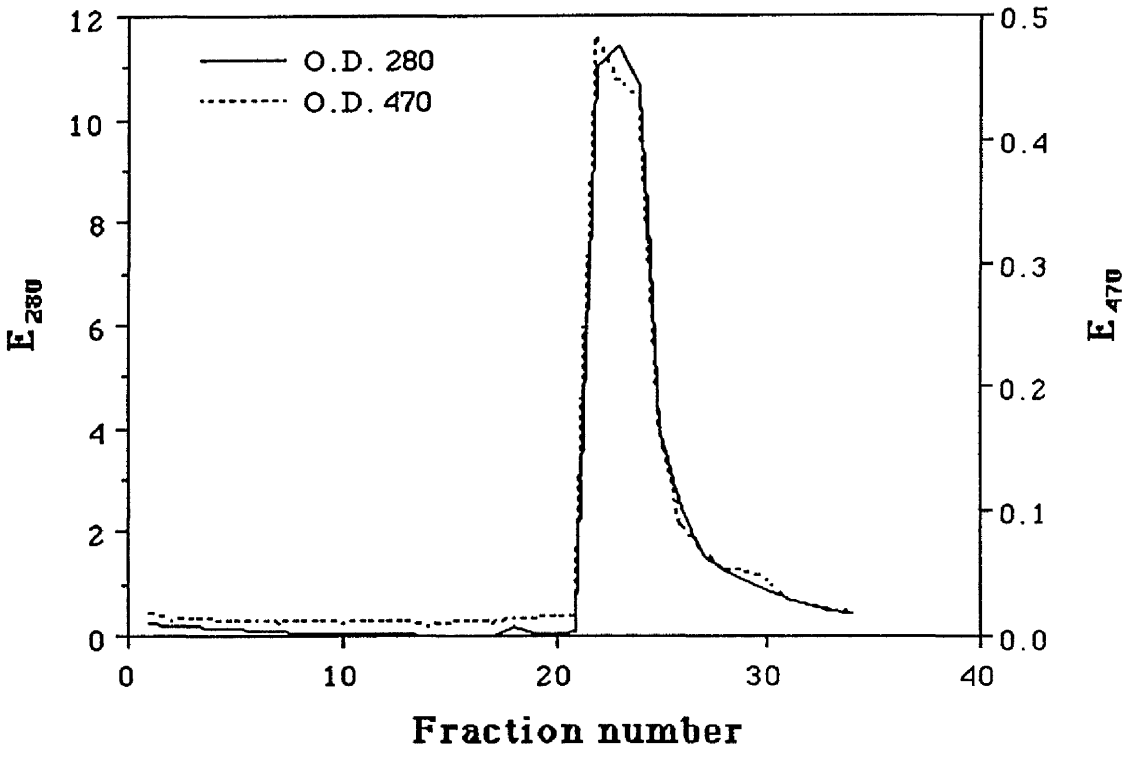

Figure 18 Optical densities of lactoferrin fractions at 470 and 280 nm eluted from a Sephadex CM 50 gel using 0.05 M Tris, pH 8.0 followed by 0.05 M Tris/2.0 M NaCl, pH 8.0.

dialysed against 3 daily changes of H_2O and freeze dried. To obtain apo-Lf, Lf was dissolved in 0.1 M acetate, 0.2 M Na_2HPO_4 and 0.04 M EDTA buffer (pH 4.0). The solution was dialysed overnight against the same buffer at 4° C. The EDTA was removed by a 24 h period of dialysis against PBS.

3.2.4 Cell line

CCRF-CEM (Foley *et al.*, 1965), a human T-leukaemic cell line was routinely grown in an atmosphere of 95% air and 5% CO_2 at 37° C in RPMI-1640 complete medium (see section 2.2.2.6) containing 10% FCS. Cells were maintained in log-phase growth at densities of 0.3 to 0.8 x 10^6/ml by replenishing the cultures by 5-fold dilutions with fresh medium every three days. The cells were incubated initially in RPMI-1640 alone for 1 h, and washed twice, prior to being used in any experiment. This was done to allow exocytosis of any endogenous Tf from the culture medium to occur, thus minimizing interference.

3.2.5 Detection of transferrin synthesis by CCRF-CEM cell line

CCRF-CEM cells (4 x 10^7) were taken from cultures 24 h after addition of fresh medium, washed 3 times in PBS, and preincubated in RPMI-1640 (w/o Cys) for 90 min before being incubated with 100 µCi ^{35}S-cysteine for 5 h. The immunoprecipitation procedure was carried out as described in section 2.3.9 with the following modifications:

1) To test samples, 10 µl of sheep α-HTf (SAPU) was added.

2) To competition samples, 30 μg of HTf was added followed by 10 μl of sheep α-HTf.
3) To control samples 8 μl of normal sheep serum was added.

3.3 RESULTS

3.3.1 Titration of phytohaemagglutinin

Mononuclear cells were prepared as described in section 3.2.1, and cultured in serum-free medium containing either 50 μg/ml of 75% saturated transferrin (serum-free) or 10% FCS. After 72 h the proliferation responses were assayed as described in section 2.3.2 for mouse cells. The optimum concentrations which induced maximum transformation were 1 and 5 μg/ml for cells cultured in serum-free medium and serum-containing medium respectively (**Fig. 19**). The ratio of the responses for cultures in serum-free medium to FCS is in accordance with previous findings with Con A in the case of mouse lymph node cells (see section 2.4.1).

3.3.2 The effect of iron saturation of transferrin on human lymphocyte proliferation

The cells were cultured at 2 x 10^6/ml in the complete medium (see section 2.2.2.6) in the presence of 1 μg/ml PHA and increasing iron saturation of Tf ranging from 0-200% at a concentration of 50 μg/ml. **Fig. 20** shows the proliferation of cells taken from three different individuals. Although there was a considerable variation between individuals, the same pattern of proliferation was seen. As the Tf saturation increased there was an increase in proliferation which reached a peak at around 100% iron saturation of the protein. Once this saturation level was exceeded, there was a noticeable decline of proliferation.

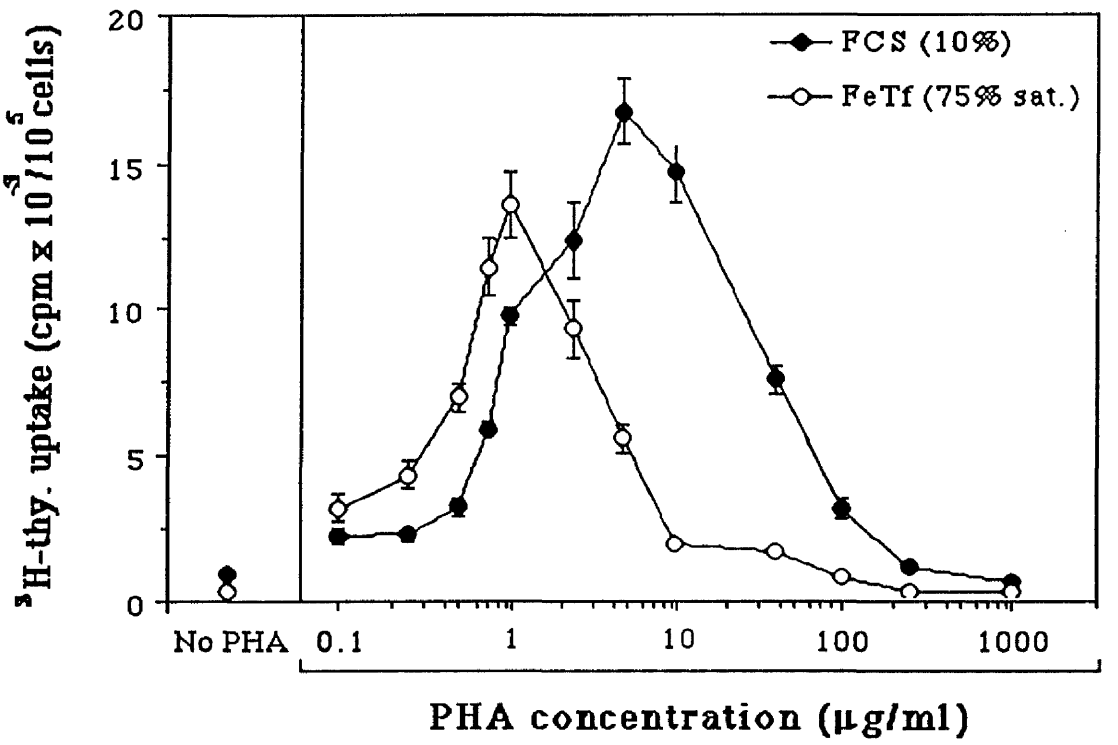

Figure 19 Titration of response to phytohaemagglutinin of human lymphocytes. Points and vertical bars represent mean counts ± SD (n = 4).

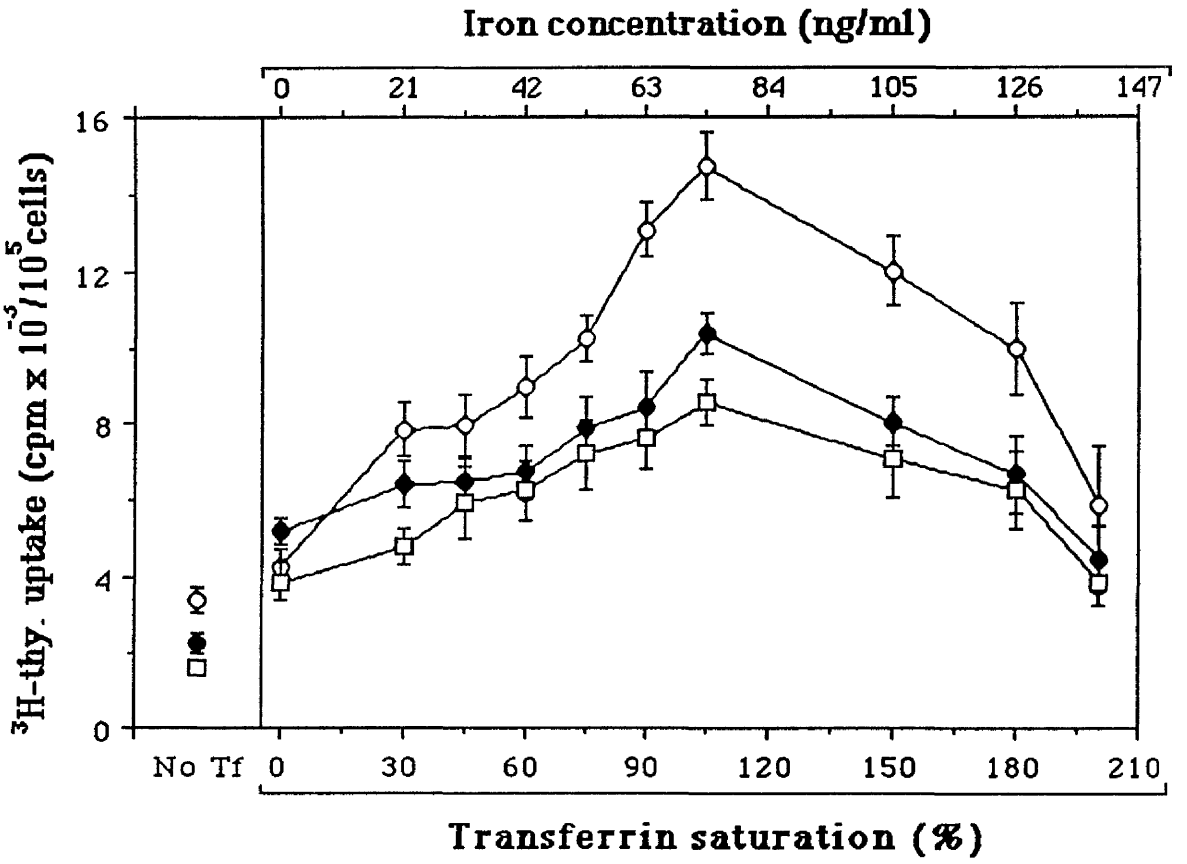

Figure 20 The effect of the degree of transferrin saturation with iron on proliferation of PHA-stimulated human lymphocytes (the graph shows proliferation of cells taken from three different donors). Points and vertical bars represent mean counts ± SD (n = 4).

3.3.3 The effect of FeNTA and FePIH on human lymphocyte proliferation

Cells were cultured at 2 x 10^6 cells/ml in the complete medium in the presence of 1 μg/ml PHA (see section 3.2.1). FeNTA and FePIH were added to the cultures at three different iron concentrations (10, 40, and 60 ng/ml). FeTf was used as a positive control with iron content equivalent to those of the two chelators i.e. iron saturations of 14, 56 and 84%. As expected, FeTf showed an enhancement of proliferation as the iron content of the protein increased up to 60 ng/ml, the equivalent of 84% saturation (**Fig. 21**). Though FePIH showed a significant enhancement as its iron content increased, it was somewhat less good than FeTf unlike the findings with mouse lymph node cells (see section 2.4.3). On the other hand FeNTA was not effective at promoting proliferation, and showed an inhibitory effect as the iron concentration was increased in the culture medium, compared with control cultures with no addition($P<0.05$ at 60 ng/ml iron).

3.3.4 The effect of iron saturation of transferrin on intracellular ferritin of proliferating human lymphocytes

The effect of Tf saturation with iron on intracellular Ft levels was investigated. Cells were cultured in serum-free medium in the presence of Tf of various iron saturations for 72 h, washed twice in PBS and resuspended at 10^6/ml in 1 ml PBS containing 0.1% BSA, 1 mM PMSF and 0.7 μg/ml pepstatin. Lysates were prepared by three cycles of

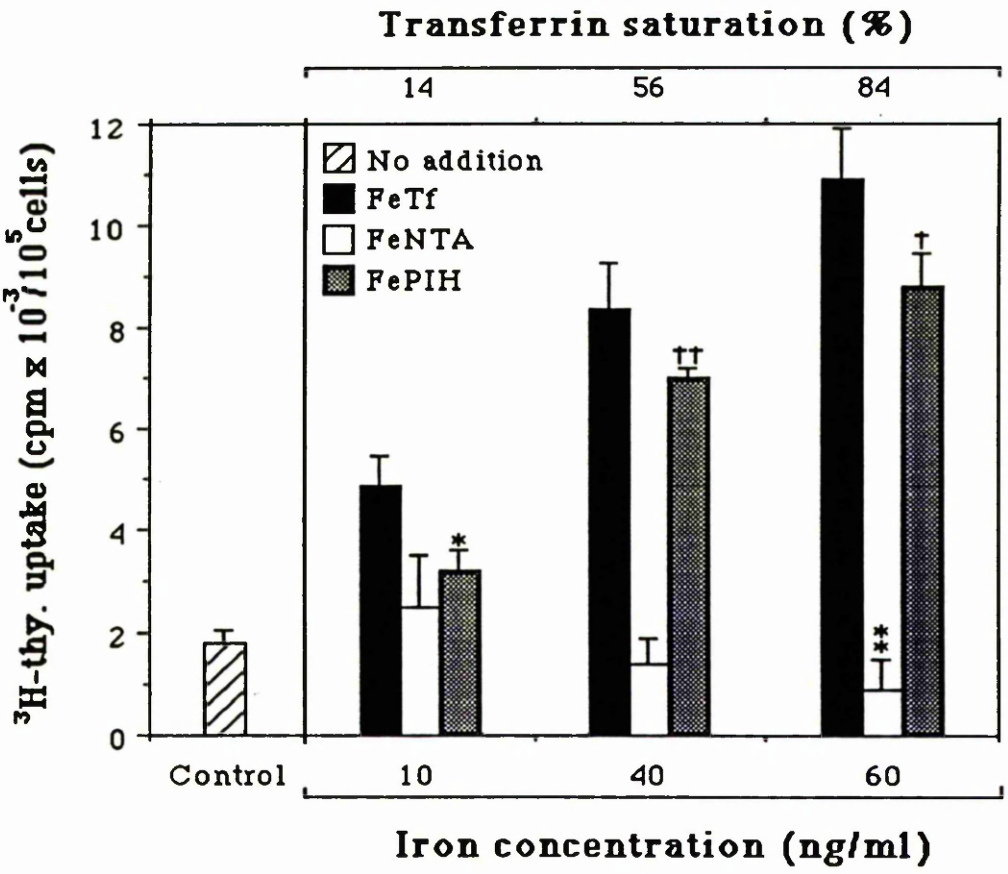

Figure 21 The effect of different iron carriers on proliferation of PHA-stimulated human lymphocytes (representative of 5 separate experiments). The columns and the vertical bars represent mean counts ± SD (n = 4). * $P<0.005$; ** $P<0.05$ compared with control (no addition), † $P<0.01$; †† $P<0.025$ compared with culture containing equivalent amount of iron bound to Tf.

freezing and thawing. Ft levels in lysates were determined by a ^{125}I radioimmunoassay method using the Magic^{R} Ft RIA kit (Ciba) which measures predominantly L type Ft (kindly performed by the Dept. of Biochemistry, Gartnaval General Hospital, Glasgow). The RIA failed to detect any Ft in lysates that were stored at -20 or -70° C. This indicates that Ft may lose its antigenicity upon storage at freezing temperature. Therefore, it was decided to test the samples straight after preparing them or storing them at 4° C overnight at the most if necessary. Cellular Ft content increased only slightly with increasing extracellular iron concentration up to the level of complete saturation of Tf where optimum proliferation was seen as judged by ^{3}H-thymidine uptake (**Table 3**), which suggests that there was some correlation between the degree of transformation and Ft synthesis. However, when this level was exceeded there was no increase in intracellular Ft levels even when extremely high extracellular iron concentrations were present in the cultures. This might indicate that like proliferating mouse lymphocytes, human lymphocytes have a limited ability to synthesise Ft in response to iron.

3.3.5 The effect of lactoferrin on human lymphocyte proliferation

ApoLf or 90% saturated FeLf were added at 50 μg/ml to cells cultured in serum-free medium (see section 2.2.2.6) containing varying iron saturations of Tf. The effect of apoLf and 90%-saturated FeLf added alone was also included and control cultures were set up with cells in medium alone.

Table 3 The effect of transferrin saturation on intracellular ferritin in proliferating human lymphocytes

	Control (No Tf)	Transferrin saturation (%)				
		0	30	90	200	3200
Ferritin ($ng/ml/10^6$ cells)*	34 ± 8	43 ± 6	67 ± 10	119 ± 14	90 ± 9	106 ± 15
^{3}H-thym. uptake ($cpm/10^5$ cells)†	3315 ± 275	4603 ± 508	8955 ±796	12588 ± 982	7943 ± 801	453 ± 34

* mean of triplicate experiments ± SD

†mean ± SD (n = 4)

Iron-loaded Lf did not have any significant effect on proliferation when added alone compared with control cultures with no addition (**Fig. 22**), which indicates that FeLf could not substitute for Tf in promoting lymphocyte proliferation. However, the iron-free form of the protein caused a significant decrease of transformation when added alone ($P<0.005$) or to cells cultured in the presence of increasing iron saturation of Tf. Apo-Lf caused a slight but significant decrease of the proliferative response of these cells up to the level of 75% iron saturation of Tf($P<0.025$, $P<0.05$, and $P<0.025$ with cultures containing apoTf, 15% and 75% saturated-Tf respectively). On the other hand, FeLf did not cause any significant change in thymidine uptake when added to cells cultured in the presence of Tf at any saturation level. However at much higher iron concentration (200%), the presence of apo-Lf in cultures markedly restored blastogenesis. At higher concentration (600%) Lf lost its protective effect as proliferation dropped below control levels. Finally, at extremely high iron concentration in the medium (3200%), all proliferation was abolished.

3.3.6 The effect of lactoferrin on intracellular ferritin levels of proliferating human lymphocytes

In view of the results of the previous section it was of interest to investigate the effect of Lf on intracellular Ft levels to see whether the possible sequestration of iron from the cells by Lf reduced synthesis of Ft. Cellular Ft content was measured as described in section 3.3.4. **Table 4** shows that while FeLf did not have any effect on intracellular Ft levels, the iron-free form of the protein caused a slight decrease

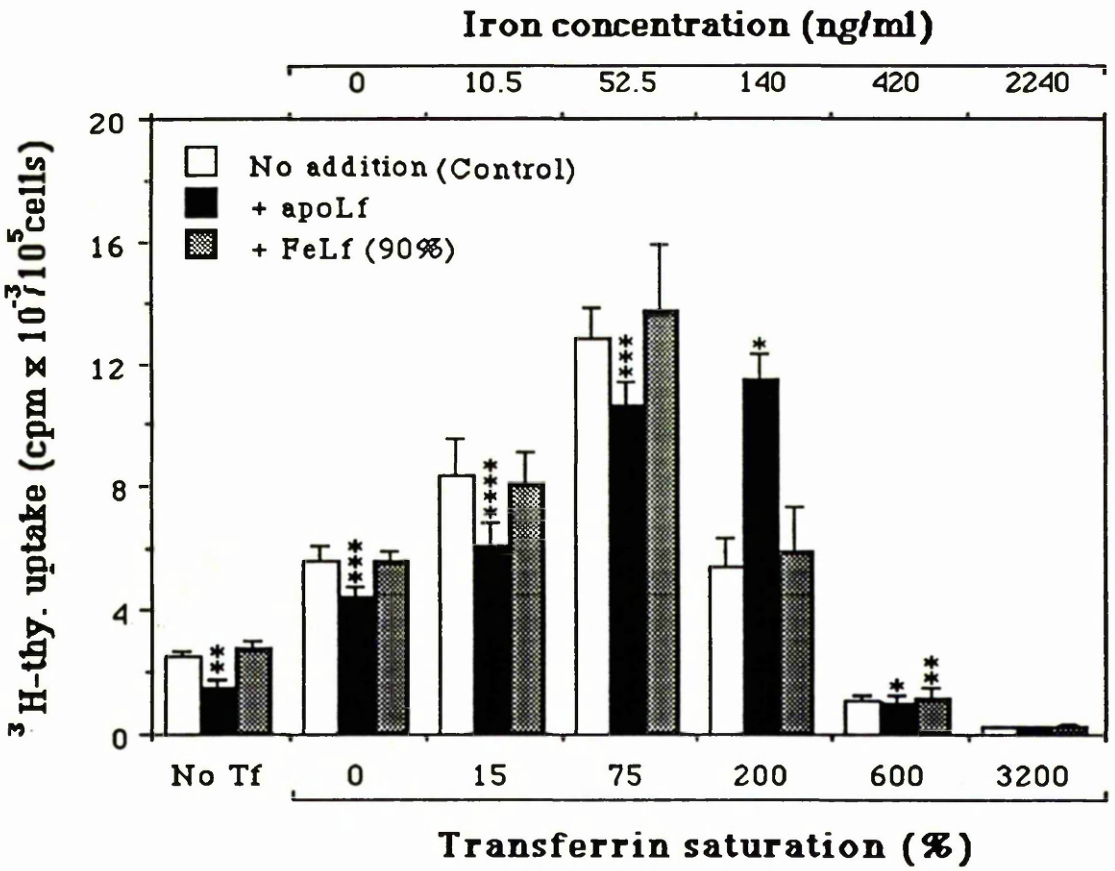

Figure 22 The effect of lactoferrin on proliferation of PHA-stimulated human lymphocytes cultured with or without transferrin (representative of 4 individual experiments). Columns and vertical bars represent the mean values ± SD (n = 4). * $P<0.0005$; ** $P<0.005$; *** $P<0.025$; **** $P<0.05$ compared with control (no addition)

Table 4 The effect of lactoferrin on intracellular ferritin in proliferating human lymphocytes

	Control (No Tf)	ApoLf	FeLf (90% sat.)
Ferritin ($ng/10^6$ cells)*	45 ± 6	29 ± 4	40 ± 8

* mean of duplicates ± SD

compared to control cultures with no addition

3.3.7 The effect of iron saturation of transferrin on T-cell subsets

The following subsets; CD3, CD4, and CD8 were determined by an indirect immunofluorescence as described in section 3.2.2. The proportion of $CD3^+$ cells in the cultures was not significantly affected by at any of the iron concentrations tested (**Table 5**). Neither apoTf nor a high concentration of iron in the medium (200%) had any effect on the proportion of these cells in the cultures compared to control cultures with no addition. However, the high level of iron (200% Tf saturation) decreased the ratio of CD4/CD8 by almost a half. This was due to a decrease in the proportion of $CD4^+$ cells and a corresponding increase in the percentage of $CD8^+$ cells compared to control cultures with no addition. In contrast, lower Tf saturations with iron and iron-free Tf did not show any effect on the CD4/CD8 ratio which remained constant as compared with control cultures with no addition. Finally at 75% saturation of Tf, the proportion of $CD4^+$ cells was significantly higher compared to control cultures with no addition.

3.3.8 The effect of FeNTA and FePIH on T-cell subsets

The effect of iron in the forms of FeNTA and FePIH and in the absence of Tf on T-subset marker expression was investigated. An iron concentration of 50 ng/ml equivalent to Tf saturated to 71% was chosen,

Table 5 The effect of transferrin saturation on expression of CD3, CD4, CD8, by proliferating human lymphocytes

	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
No addition	64.8±5*	40.4±5	19.2±2	2.1
Apo Tf	66.2±6	43.0±4	21.1±3	2.0
FeTf (30%)	71.8±5	51.2±6	23.3±3	2.2
FeTf (75%)	73.0±6	53.2±6••	23.7±6	2.2
FeTf (200%)	64.1±7	33.3±3•••	27.9±3•	1.1

*mean of percentage of +ve cells ± SD (n = 4) (representative of 3 separate experiments). •$P<0.01$; ••$P<0.02$; •••$P<0.05$ compared to control (no addition).

and 71% saturated Tf was also included as a positive control. As it was seen in previous section with non-Tf bound iron in the presence of iron loaded Tf, iron as FeNTA lowered the ratio of CD4/CD8 cells (**Table 6**).This was the consequence of a considerable decrease of the proportion of $CD4^+$ cells and an increase of the percentage of $CD8^+$ cells compared to control cultures with no addition. FePIH did not have any significant effect on the ratio of CD4/CD8 as compared with control. On the other hand, the proportion of $CD3^+$ cells in the presence of FeNTA in cultures was not significantly different from those containing FeTf or FePIH. The results obtained with 79% saturated Tf gave fairly similar results as compared to the previous experiment.

3.3.9 Proliferation of CEM cells in the presence of different iron saturations of transferrin

The cells were cultured in conical test tubes at 5 x 10^4 cells/ml in RPMI-1640 containing 1 mg/ml HSA in the presence of different iron saturations of Tf ranging from 0 to 3200% for 24 h. The degree of transformation was assessed as described in section 2.3.2 for normal lymphocytes. First of all it is of interest to notice the relatively high proliferative background of cells cultured in medium without Tf (**Fig. 23**). Nevertheless the addition of apo-Tf caused a noticeable increase in the rate of proliferation. At lower iron saturations of Tf proliferation of CEM occurred at almost the same rate with very little variation up to 200%. In the presence of larger amount of iron up to 1200%, a gradual decrease in proliferation occurred as the concentration of iron increased. Nevertheless good proliferation was still maintained even at this high level of iron. Only when this level was exceeded did proliferation drop

Table 6 The effect of Fe-transferrin, FeNTA, and FePIH on expression of CD3, CD4 and CD8 by proliferating human lymphocytes

	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
No addition	68.8±6*	43.4±4	20.5±3	2.1
FeTf (50 ng/ml Fe) (=71% sat.)	71.1±5	51.3±5•••	23.2±4	2.2
FeNTA (50ng/ml Fe)	66.6±6	31.2±4•	27.9±3••	1.1
FePIH (50 ng/ml Fe)	68.8±8	47.4±3	22.7±4	2.1

* mean of percentage of +ve cells ± SD (n = 4) (representative of 3 separate experiments). •$P<0.01$; ••$P<0.02$; •••$P<0.05$ ccompared to control with (no addition).

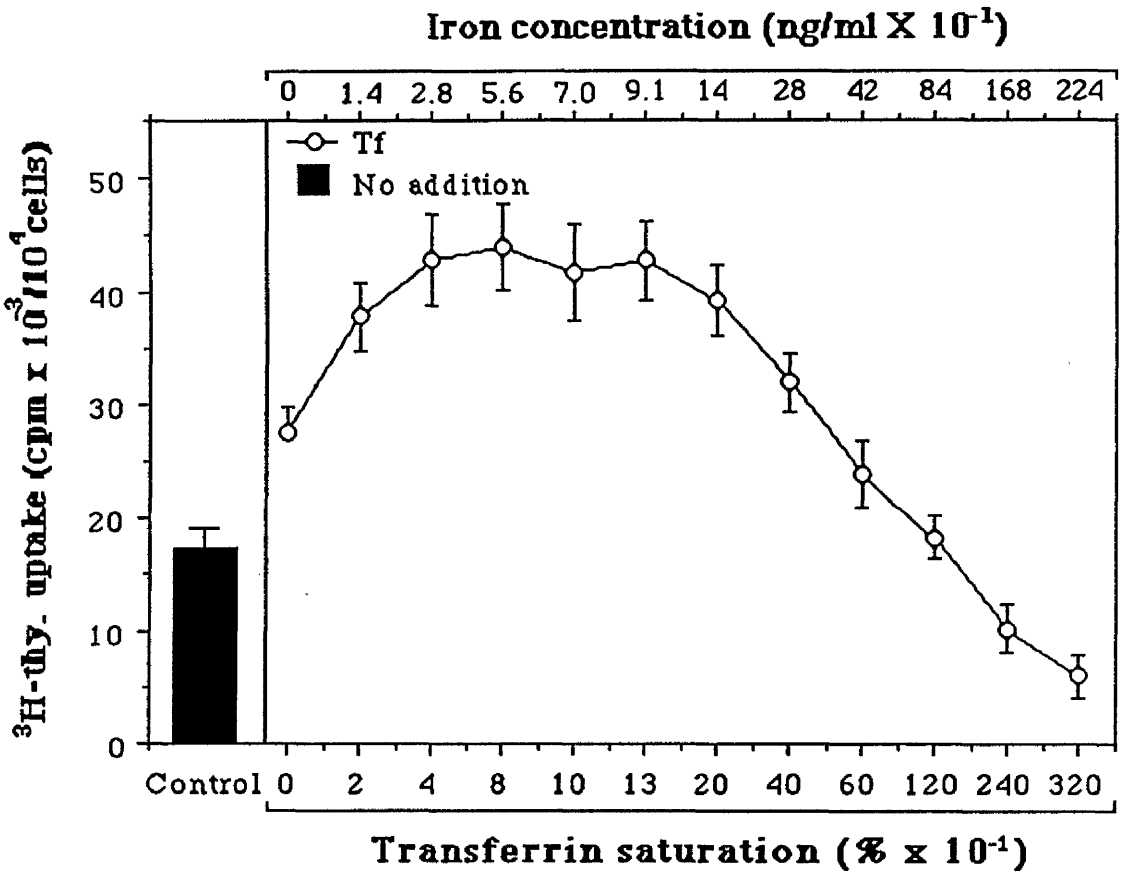

Figure 23 The effect of transferrin saturation on CCRF-CEM cell growth (representative of 5 separate experiments). Columns and vertical bars represent mean values ± SD (n = 4).

below that of the control without Tf.

3.3.10 Proliferation of CEM cells in the presence of FeNTA and FePIH

Cultures containing 5 x 10^4 cells/ml were set up in RPMI-1640 containing 1 mg/ml HSA in the presence of FeNTA and FePIH with increasing iron concentrations ranging from 10 ng/ml to 1 μg/ml which is equivalent to a range of Tf saturation from 14 to 1429%. Cultures containing these iron saturations of Tf were included. Both chelates promoted good proliferation compared to controls without addition (**Fig. 24**). Indeed, there was no significant difference between either chelates and FeTf in supporting proliferation, indicating that the two chelates are equally effective at promoting proliferation of CCRF-CEM cells as FeTf. Surprisingly, the rate of proliferation did not drop below the control levels at high iron concentration, which indicates that these cells could handle very large amounts of iron in both physiological and non-physiological forms, perhaps due to a greater ability to increase Ft synthesis.

3.3.11 The effect of iron saturation of transferrin on intracellular ferritin of CCRF-CEM cells

The effect of Tf saturation with iron on intracellular Ft levels in CCRF-CEM was examined to investigate whether these cells are able to show active response to iron by increasing Ft synthesis. Cells were

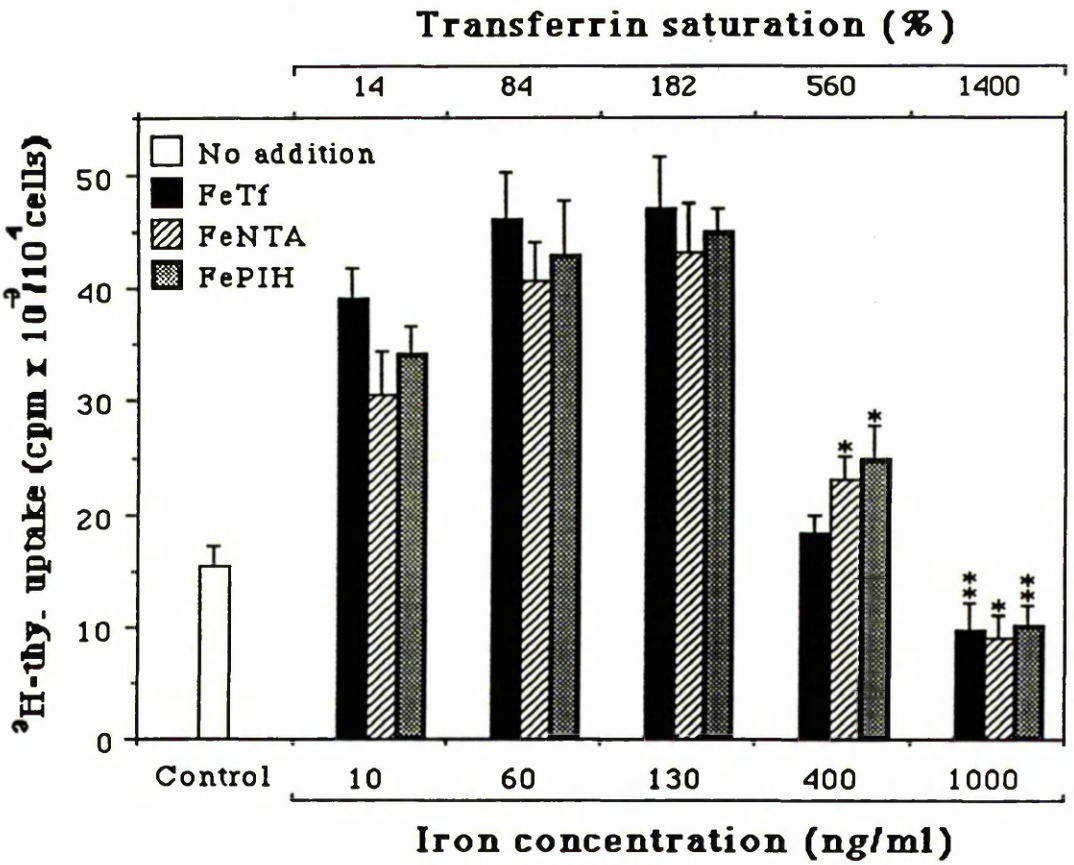

Figure 24 The effect of different iron carriers on CCRF-CEM cell growth (representative of 4 separate experiments). Columns and vertical bars represent the mean values ± SD (n = 4). * $P<0.005$; ** $P<0.01$ compared with control (no addition).

cultured in serum-free medium in the presence of Tf of various saturations for 24 h. The Ft levels were assayed as described in section 3.3.4 for normal human lymphocytes. Surprisingly, Ft content of cells (**Table 7**) or at least L-rich type Ft content at all Tf saturations tested was extremely low (in the order of 10-25 X less compared to normal human lymphocytes, see **Table 3**) and instead of showing as expected a marked increase of intracellular Ft content as iron concentration was increased in the culture medium the cells did not show any increase whatsoever.

3.3.12 Proliferation of CEM cells in the presence of lactoferrin

Cells were cultured at 5 x 10^4 cells/ml in RPMI-1640 containing 1 mg/ml HSA. ApoLf or 90% saturated Lf (50 μg/ml) were added either alone or to cultures containing FeTf or FCS. As was found with normal human lymphocytes apoLf and FeLf did not have any effect on the proliferation of CEM cells when added alone (**Fig. 25**). ApoLf was only inhibitory when added to cultures containing FeTf ranging from 0 to 75% saturation, while beyond this level of saturation up to 200% and with cultures containing FCS, it did not have any effect. In contrast at higher iron saturation (600%), apoLf has a slight but significant restorative effects on the modest inhibitory effect of high iron concentration on the proliferation of CCRF-CEM cells($P<0.01$).

Table 7 The effect of transferrin saturation on intracellular ferritin in CCRF-CEM cells

	Control (No Tf)	Transferrin saturation (%)				
		0	75	200	400	800
Ferritin ($ng/10^6$ cells)*	3.5 ± 0.5	2.0 ± 0.3	3.0 ± 0.1	4.5 ± 0.6	4.2 ± 0.4	3.9 ± 0.7

* mean of duplicates ± SD

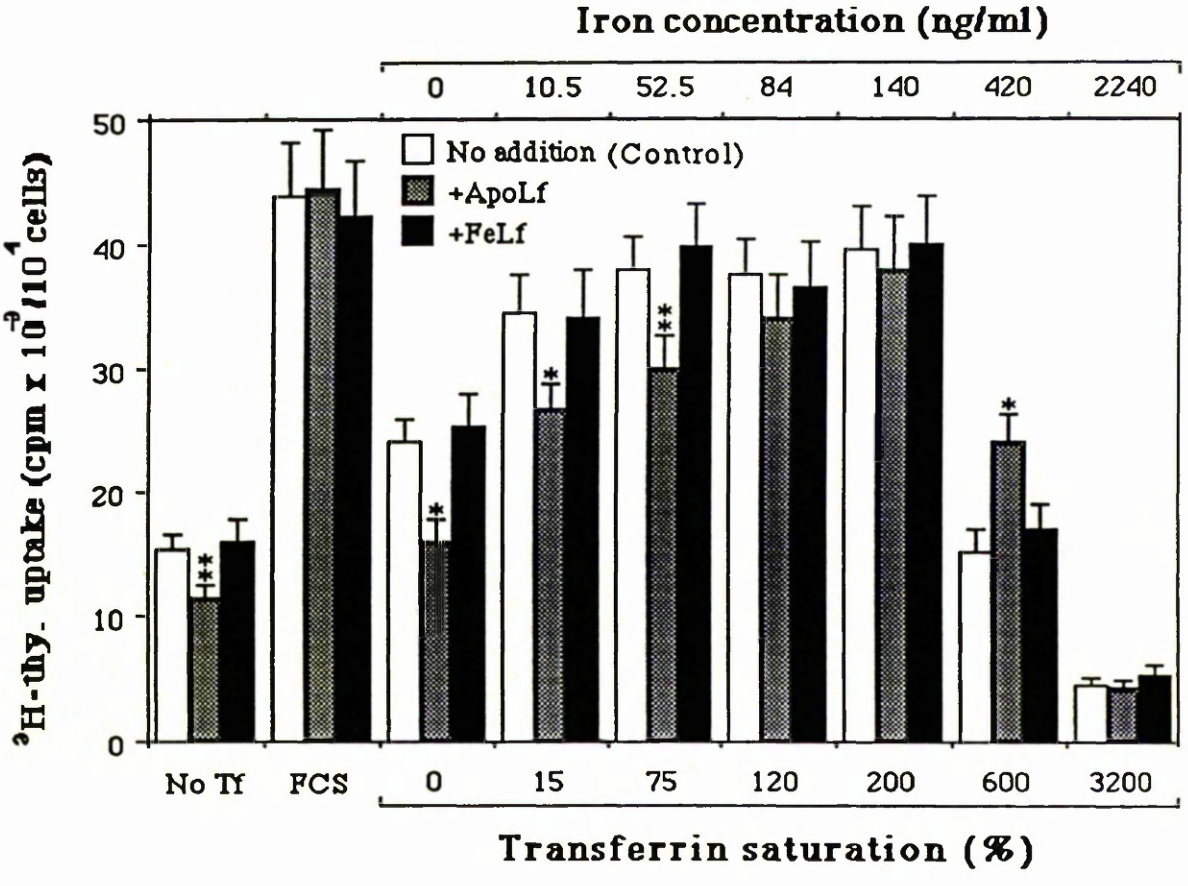

Figure 25 Effect of lactoferrin on growth of CCRF-CEM cells cultured with or without transferrin (representative of 5 separate experiments). Columns and vertical bars represent the mean values ± SD (n = 4). * $P<0.001$; ** $P<0.01$ compared with control (no addition).

3.3.13 Detection of transferrin synthesis by CEM cells

CCRF-CEM in logarithmic phase, i.e from cultures 24 h after subculturing, were subject to the immunoprecipitation method for detection of Tf synthesis as described in section 2.3.9. **Fig. 26** clearly shows that these cells, which were cultured before the experiment in serum-containing medium, have the ability to synthesise Tf. The disappearance of the band corresponding to Tf when excess cold Tf was added to the system to compete with the newly synthesised radiolabelled Tf, or when the anti-serum was omitted demonstrate specificity of the assay

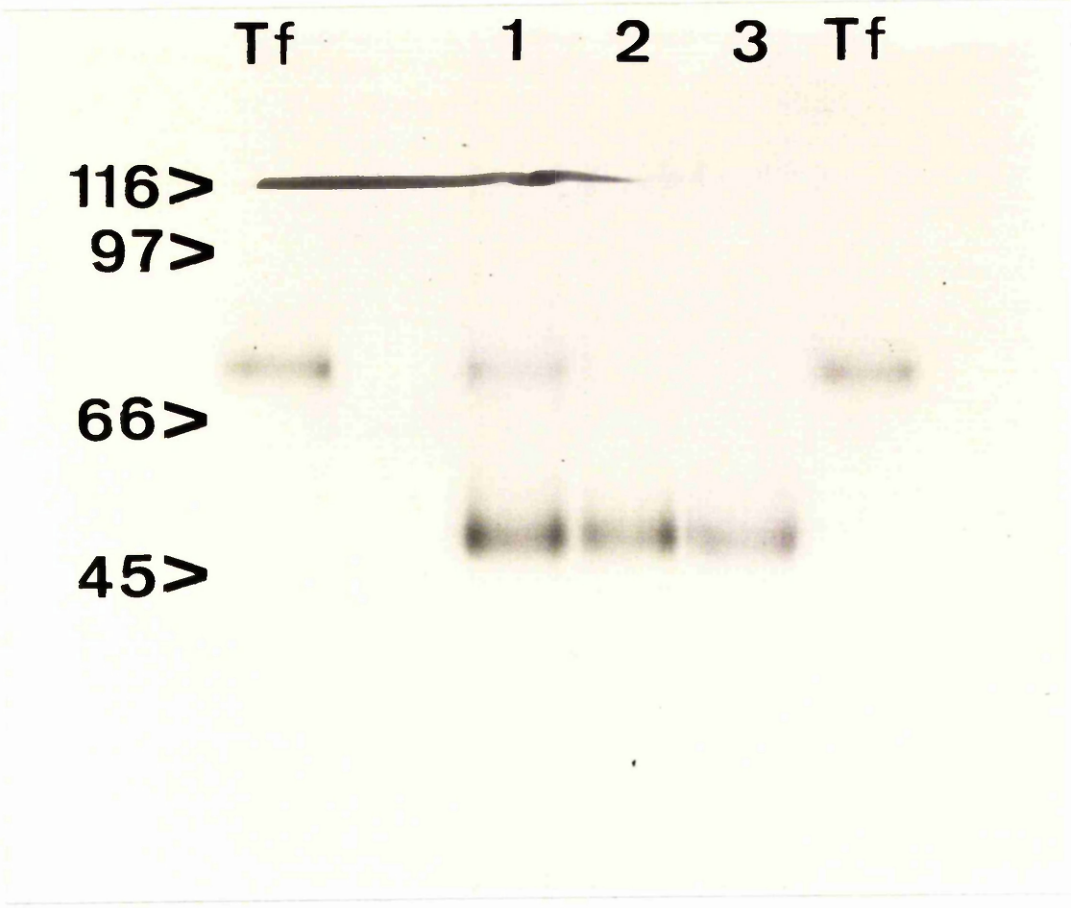

Figure 26 Autoradiography of ^{35}S-cysteine incorporated into transferrin by CCRF-CEM cells

1: *Test* (+ sheep α-human transferrin)
2: *Competition* (+ human transferrin, + sheep α-human transferrin)
3: *Control* (+ normal sheep serum)

3.4 DISCUSSION

The work reported in this chapter has attempted to push the investigation of the action of iron on lymphocytes further on by looking at its effect at different levels and forms on human lymphocyte transformation *in vitro* in comparison to mouse lymphocytes and a T-leukaemic cell line. Non-Tf bound iron in the form of FeNTA was shown to inhibit murine lymphocyte transformation, probably because of the inability of these cells to respond to iron by increasing Ft synthesis. To confirm and extend some of these effects seen on mouse lymphocytes, and to establish whether these findings apply to the normal human cells and their transformed counterpart is one of the aims of this chapter. It also attempts to define more closely the differential effect of iron on different T-cell subsets. The effect of another related iron-binding protein, Lf, was also investigated

3.4.1 The effect of iron saturation of transferrin, FeNTA, and FePIH on proliferation of human lymphocytes

There was a considerable variation in the absolute figures of the proliferative response between individuals. Despite this the same pattern of proliferation in the presence of increased iron saturation of Tf was obtained. Variability in human lymphocyte responses to mitogens have been reported by other investigators (Fan *et al.*, 1977; Platz *et al.*, 1976), and the presence of this variability has become an accepted problem in the clinical evaluation of human lymphocyte mitogen

responses. As in the case of mouse lymph node cells (section 2.4.2), the degree of transformation of human peripheral lymphocytes upon PHA stimulation cultured in medium containing apo-Tf (in practice 6-8% saturated) was lower compared with the response of the cells cultured in medium containing higher iron saturations of Tf, up to around 100% saturation. These results confirm the previous findings with mouse lymph node lymphocytes that one of the important factors in controlling the proliferative response of lymphocytes *in vitro* is the content of transferrin-bound iron in the culture medium.

As far as the effect of the chelators on human lymphocyte proliferation is concerned, it was found that as with mouse lymphocytes, iron-NTA cannot substitute for iron-Tf and at relatively high concentrations actually inhibits proliferation, but chelation with PIH was more effective. Nevertheless, the results suggest that iron taken up in this form may be used immediately by the cell and permit an increase in the cellular metabolic response to mitogens. Overall, the findings with human lymphocytes were broadly similar to the one obtained using the mouse system. However, the enhancing effect of both FeTf and FePIH on human lymphocytes was less than the effect on mouse cells. This was by almost a third (~600% increase at optimum saturation of Tf compared to control with mouse cells against ~400% with human cells). This is possibly due to the ability of human (Lum *et al.*, 1986), but not mouse lymphocytes to make their own Tf, or at least to the possibility that the former could be actively engaged in synthesising this protein while the latter are less, as it is discussed in section 2.5.4. The same may be true in the case of FePIH which was not quite as efficient compared to its

effect on mouse lymphocytes.

Surprisingly, human lymphocyte seems to be more resistant to the inhibitory effect of FeNTA compared to murine cells, knowing that the latter were taken from the blood of these animals where iron saturation of the transferrin is known to be higher compared with humans. While FeNTA at 40 ng/ml iron did not have any significant effect on human lymphocyte, this concentration lowered the proliferative capacity of mouse lymphocytes by 56% as compared to control. However, when this concentration was increased to 60 ng/ml the proliferation of both mouse and human cells was affected, but only by 46% with human cells compared to 80% in the mouse one.

3.4.2 The effect of iron saturation of Tf on intracellular ferritin levels of proliferating human lymphocytes

Since lymphocytes are not specialized for iron storage, the sequestration of iron by Ft might serve a detoxification function. Unlike other cell types, mouse lymphocytes seem generally not to have any marked increase in intracellular Ft levels in response to iron presented in different forms (see section 2.4.6). To confirm this finding and to extend it to human lymphocytes, it was decided to investigate this in more detail. In the experiments with mouse cells, just two different concentrations of iron were used for each form of iron. In the present chapter the relationship between iron availability (in the form of increasing iron saturation of Tf) and cellular Ft content was looked at.

Ft synthesis by human lymphocytes cultured in the presence of Tf

increased only modestly with increasing intracellular iron concentration. This confirms previous evidence with mouse lymphocytes that these cells have a limited capacity to synthesise Ft in response to iron. However in the present study there was a suggestion that only Tf bound iron is capable of stimulating Ft synthesis since there was an increase as the saturation of Tf present in the culture medium increased up to around the complete saturation of Tf present. Thereafter, when all Tf present was loaded with iron, exposure of proliferating lymphocytes to non-Tf bound iron by further increasing the iron concentration did not show any further increase in Ft levels. Therefore, iron in a "free" form was not able to stimulate Ft synthesis in human lymphocytes. Alternatively, it could also be that Ft synthesis was already maximal with 100% saturation of Tf. These findings agree with the suggestion of Taylor *et al* (1987) that iron bound specifically to Tf does stimulate Ft synthesis in lymphocytes. Phillips and Rutledge (1984) have also reported that FeTf increased Ft synthesis. Recently Pattanapanyasat (1989b) has reported that addition of increasing levels of FeNTA to PHA-stimulated lymphocytes caused an overall increase in intracellular Ft levels. However in this study the cells were cultured in medium containing 10% FCS. The high concentration of partially saturated bovine Tf in the system used is capable of binding iron from FeNTA, resulting in an increased saturation of this Tf, which could explain the observed increase in cellular Ft content. The increase of Ft levels seen in the present study could be due to the effect of iron on the rate of Ft catabolism. Pattanapanyasat *et al* (1988) have suggested that iron may decrease the rate of catabolism thereby increasing cellular Ft content in response to iron. It is well established that monocytes show a higher intracellular Ft concentration than lymphocytes, and that these

cells exhibit an increase in Ft synthesis in vitro in response to iron in the culture medium (Summers *et al.*, 1975; Dörner *et al.*, 1983b). Therefore, the increase in Ft levels observed in the present study could be due to the adherent cell population not being separated out.

The apparent insensitivity of lymphocytes to iron present in the medium when in excess of the binding capacity of Tf suggests that these cells have a different control mechanism for Ft synthesis to other types of cells which are known to respond to much higher levels of iron bound to different carriers. It seems possible that in lymphocytes the stimulation of Ft synthesis by iron is normally at its maximum when all the iron present in the medium saturated all the Tf present, after which a balance between synthesis and degradation or conversion to Hs would result in an equilibrium being attained. In the initial process of degradation Ft protein loses its immunoreactivity (Jacobs *et al.*, 1978) and can no longer be measured by the specific assay used here. Stimulation of lysosomal uptake by an increasing Ft load might result in a relatively short life of an individual Ft molecule and could account for the decline in the intracellular Ft levels seen. Worwood *et al* (1984) showed that peripheral blood mononuclear cells cultured *in vitro* accumulate Ft very rapidly, reaching a plateau after 10 h incubation, with little or no further increase in Ft occurring after 20 h.

The proliferation results show a good correlation with the Ft levels up to around the full saturation of Tf. At this point the proliferation response was at its maximum. However at higher iron concentrations the proliferation response dropped while cellular Ft content remained constant. This could be interpreted as demonstrating the protective effect

of Ft synthesis and the onset of iron toxicity when it fails to match levels of iron donated to cells

3.4.3 The effect of lactoferrin on proliferation and ferritin levels of human lymphocytes

Lf, another member of the Tf family is an important constituent of the secondary granules in neutrophils. During an inflammatory response Lf is released from lysosomes and is readily detectable in inflammatory fluids (Bennett and Skosey, 1977). Thus, Lf might modulate the cellular immune events associated with inflammatory responses.

Previous studies have demonstrated a regulatory role for Lf. It has been shown that human Lf binds to monocytes and macrophages (van Snick and Masson, 1976; Bennett an d Davis, 1981; Goavec *et al.*, 1985; Oria *et al.*, 1988), and that the protein was internalized and degraded by the cells which incorporated iron into Ft (van Snick *et al.*, 1977). Furthermore, it has been claimed that a specific receptor exists on monocytes (Birgens *et al.*, 1983; Bartal *et al.*, 1987). Lf has also been found to play a regulatory role in the immune responses involving lymphocytes. The primary *in vitro* antibody response of murine spleen cells to T-dependent and independent antigens is suppressed in the presence of Lf (Duncan and McArthur, 1981). Recently, Mazurier *et al* (1989) have showed that Lf binds to a specific receptor on lymphocytes, and that these receptors are only expressed on the surface of actively proliferating cells, and unlike Tf receptors they are not expressed intracellularly in resting cells. These claims of the existence of specific

receptors for Lf by these studies are open to doubt due to the fact that Lf is highly cationic and binds to a wide range of proteins and other molecules (Hekman, 1971).

There are conflicting reports concerning whether Lf can support proliferation, not only in studies dealing with different types of cells but also among those which dealt exclusively with lymphocytes. In this work neither iron-free nor iron-loaded Lf had any effect on PHA-stimulated human lymphocytes as compared to control cultures with no addition, and was thus found to be incapable of exhibiting Tf-like activity when added alone. Previous investigations have shown that colostrum suppresses mitogen and alloantigen-stimulated lymphocyte proliferation (Crago *et al.,* 1981; Richie *et al.,* 1981). It has been reported that Lf released from phagocytosing neutrophils inhibits transcriptional activity in mixed lymphocyte culture and PHA-stimulated human lymphocytes (Slater and Fletcher, 1987). On the other hand, Mazurier *et al* (1989) have described Lf as a growth stimulating factor for PHA-stimulated lymphocytes. These latter findings must be taken very cautiously since up till now no evidence whatsoever has been advanced to demonstrate an ability of Lf to donate iron to lymphocytes, despite their claim that lymphocytes exhibit a receptor for Lf. However as mentioned above the nature of the binding sites for Lf is still controversial and many authors believed that they encompass a variety of molecular species (non-specific adsorptive endocytosis) as was reported in the case of macrophages (Regoeczi *et al.,* 1985; Moguilevsky *et al.,* 1985).

The effect of Lf on the proliferation of lymphocytes cultured in the presence of increasing saturation of Tf with iron was examined to see

whether Lf, which has a much higher affinity for iron than Tf (Bezkorovainy and Zschocke, 1974), could help in restoring the ability of lymphocytes to proliferate in the presence of excess non-Tf bound iron.

First of all it is worth noting that apoLf significantly inhibited proliferation of lymphocytes as compared to control cultures when added alone($P<0.005$). When added to cultures containing lower iron saturations of Tf (15-75%) the iron-free form of the protein was slightly inhibitory to proliferation of cells. These results are in accordance with the finding of Richie *et al* (1987), who showed that while iron-free Lf inhibited proliferation of mitogen-stimulated human lymphocytes cultured in serum containing medium, and of mixed lymphocyte cultures, iron-saturated Lf failed to inhibit mitogen-induced proliferation. This suggests that the mechanism of suppression involves the chelating property of Lf. What are the possible mechanisms involved in this inhibitory activity? Is it by mere retention of the metal at the extracellular level or, knowing that some reports have claimed that lymphocyte exhibit specific receptors for the protein (Mazurier *et al.*, 1989) might this involve interference with the intracellular iron pool?

Apo-Lf could compete with Tf in the medium for iron, resulting in the deprivation of cells of iron and thus preventing them from proliferating. By removal of iron from Tf in the extracellular environment, Lf makes iron unavailable, and it is not likely to deliver iron to these cells as discussed above. One could also speculate that Lf competes for the intracellular iron of these cells. Lf might be able to enter the cell and compete for iron, and having bound this iron, there are

two possibilities. If the protein has the ability to cross the cell membrane to the extracellular environment, it could shift iron from the intra- to the extracellular milieu, or alternatively it could accumulate within the cell as an inactive form of iron. In both cases, apo-Lf would make iron unavailable to the cells rather than preventing its uptake. It is also possible that the inhibitory effect of Lf is a result of the combination of both mechanisms.

To confirm this view it was of interest to examine the effect of Lf on the intracellular iron reserves of the cells i.e its effect on cellular Ft levels. Apo-Lf was found to decrease intracellular Ft levels compared with the control, while 90%-saturated FeLf had no effect. The effect of Lf on intracellular Ft levels is analogous to the effect of iron chelates on Ft synthesis. Summers *et al* (1975) observed that chelation of iron with DFO markedly reduced lymphocyte Ft synthesis *in vitro*, and suggested that mechanisms maintaining normal basal levels of cellular Ft synthesis are iron-dependent. However, this seems to be in opposition to the earlier findings (see previous section 3.3.4) that excess iron failed to stimulate more Ft synthesis. It could be that under normal iron conditions the response is maximal, and that the presence of a chelator causes a decrease in Ft synthesis while addition of iron increases it.

On the other hand, in cultures containing Tf at a higher degree of saturation (200% saturation) the inhibitory effect of non-Tf bound iron was reversed by the presence of apo-Lf in the culture medium, where it was markedly stimulatory. In sharp contrast, the iron-loaded form of the protein had no effect whatsoever on lymphocyte proliferation either when added alone or when added to lymphocytes cultured in the presence of

varying saturations of Tf with iron. In the presence of non-Tf bound iron, apoLf, probably binds this excess and neutralizes its toxic effect on cells, resulting in restoration of cell proliferation. Indeed, Ambruso and Johnston (1981) showed that iron-poor Lf (apparently not completely depleted of iron) inhibited hydroxyl radical production. Similarly, using lipid peroxidation as an indicator of hydroxyl radical activity, Gutteridge *et al* (1981) showed that partially saturated Lf (20%) has antioxidant properties in that it can inhibit the catalytic action of iron salts, but iron saturated Lf had no effect. All this suggests that one of the functions of unsaturated Lf which never occurs completely saturated under normal physiological conditions is to dampen these potentially toxic reactions.

The above results are comparable to the well established function of Lf in human milk as an inhibitor of bacterial growth by chelating iron and preventing its availability to microorganisms (Bullen *et al.*, 1972; Arnold *et al.*, 1977; Bullen, 1987). However another possibility is that the inhibitory effect of apoLf on lymphocyte proliferation could involve more than one mechanism. Lf may possess other non-iron related intrinsic properties which in turn cause other biological effects that could affect lymphocyte proliferation indirectly. Among these effects are inhibition of colony stimulating activity (CSA) production, which results in decreased release of cytokine(s) from monocytes which are responsible for triggering other cells such as T-lymphocytes to release other stimulating factors (Broxmeyer *et al.*, 1976, 1978; Bagby *et al.*, 1981, 1983). This suggests that Lf might mediate its effect through suppression of IL-1 production (Smith *et al.*, 1980; Zucali *et al.*, 1987; 1989), which has numerous immunological activities, including triggering the release of the T-cell growth factor IL-2 from T-lymphocytes

(Dinarello, 1984). Slater and Fletcher (1987) have also reported that Lf inhibits uridine uptake in allogenic mixed lymphocyte reactions, by suppressing the production and release of a soluble factor subsequently identified as IL-2 (Slater and Fletcher, 1987).

In summary at very high iron saturation of Tf, the presence of apo-Lf in the medium contributed to avoiding the deleterious effect of non-Tf bound iron on proliferation probably by sequestering the metal, resulting in prevention of the decline of proliferation seen when high levels of iron were present. At the same time the excess iron loaded Lf and thus prevented there being any inhibitory effect of iron-free Lf, either on lymphocytes directly or on other accessory cells.

3.4.4 The effect of iron saturation of transferrin, FeNTA and FePIH on the expression of T-cell surface markers

In the previous experiments it was shown that excess iron in the presence or the absence of iron-loaded Tf affects T-lymphocyte transformation *in vitro*, It might be possible that iron also affects the immune response by altering the relative populations of T-lymphocyte populations and subpopulations.

To examine this aspect of immunoregulatory properties of iron, evaluation of its effect on the expression of lymphoid surface markers on actively dividing lymphocytes was carried out. Human lymphocytes were chosen to perform this study because of the availability of the monoclonal antibodies to the well known different subset markers of these cells. The phenotypic study reported in the present study, revealed that iron

interferes with expression of two cell surface antigens required for cell activation i.e CD4 and CD8. The presence of iron not bound to Tf caused a decrease in CD4/CD8 ratio, due mainly to depression of the proportion of $CD4^+$ cells and a slight increase in the proportion of $CD8^+$ cells. The same findings were seen when cells were exposed to iron in the form of FeNTA and in the absence of Tf. However, unlike FeNTA, iron in the form of FePIH did not lower the CD4/CD8 ratio. These findings are relevant to the results of the effect of iron on transformation. A likely explanation of the overall decrease in T-lymphocyte proliferation following polyclonal stimulation when cells were exposed to non-Tf bound iron in the form of FeNTA (see section 3.3.3) is that this iron affects the clonal expansion of $CD4^+$ cells which may be more vulnerable to the oxidative stress of iron resulting in oxidative injury to these cells. Allan *et al.* (1986) have demonstrated that T-lymphocytes are more susceptible to oxidant induced killing than non-T-cells. Therefore the number of the daughter cells generated in these cultures at the end of the incubation period would be less than in cultures where cells were exposed to Tf-bound iron or FePIH. These results are in agreement with findings of Good *et al* (1986) who reported that Fe^{3+} reduced both the cloning efficiency of human $CD4^+$ precursor lymphocytes and the rate of clone growth of the T-cells that did proliferate. Among T-subsets, Bryan *et al* (1986) have shown a selective effect of high concentrations of chelatable iron on $CD4^+$ cells, consisting of a decrease in the proportion of this subpopulation. It has also been reported that fewer helper precursors were present in the spleen of iron-loaded mice (Dialynas *et al.*, 1983). On the other hand, Carvalho and de Sousa (1988) were not able to show any alteration of CD3, CD4, CD8, CD1, CD22, CD10, and HLA-DR surface markers by iron, and the only molecule that was found to be

affected was the CD2. The discrepancy with this study could lie in the difference of the state of cells studied since they assessed non-proliferating cells.

One explanation of the differential effect of high iron concentrations on T-cell subsets is that it may be that in these conditions $CD4^+$ cells accumulate iron intracellularly more rapidly than the $CD8^+$ cells with toxic consequences. Alternatively, the cells may take up the iron at an equal rate, but the CD8 cells may be able to process this iron more efficiently, thus preventing accumulation of excess iron. It has been reported that among T-subsets, helper cells may be relatively sensitive to oxidant injury and that in the presence of DFO injury did not occur and proliferation was enhanced (Sagone *et al.*, 1984).

3.4.5 Proliferation of CCRF-CEM cells in the presence of transferrin, FeNTA, FePIH, and lactoferrin

Among the many differences between normal and transformed cells as studied in cell culture are their requirement for serum in promoting growth and their response to serum growth factors. Many transformed cells have less need for serum for optimal growth than untransformed cells (Sporn and Todoro, 1980). An autocrine growth regulation hypothesis which proposes that malignant cells produce and secrete hormone-like substances that induce further autonomous proliferation, has been postulated as a possible mechanism leading to malignant transformation and was studied in several tumour cell systems (Todaro *et al.*, 1976). Some transformed cells which proliferate in defined serum-

free media do not require Tf (Fernandez-Pol, 1978; Zwiller *et al.*, 1982; Basset *et al.*, 1986; Taetle *et al.*, 1985). It is therefore possible that some transformed cells must be able to regulate iron acquisition by a mechanism different from Tf-mediated uptake. Therefore it was of interest to look at the differences in the capacity of CCRF-CEM cells, a T-leukaemic cell line to utilize iron in different forms and to investigate whether these cells behave differently from their untransformed counterparts.

Unlike mouse and normal human lymphocytes in which proliferation in the presence of Tf was found to be a function of the bound iron up to 100% saturation of Tf, CCRF-CEM cells did not show any difference in their proliferative rate at different saturations of Tf up to 100% saturation. Moreover, at higher levels of iron (100-600% saturation), when non-Tf bound iron was present in the culture medium, CCRF-CEM cells were able to achieve good proliferation in contrast to normal mouse and human lymphocytes. The same was true with the chelate FeNTA which unlike normal lymphocytes had the same stimulatory effect on proliferation as Tf. As with normal lymphocytes FePIH was efficient in supporting proliferation of CCRF-CEM cells. These results are in agreement with the findings mentioned above that some iron-containing compounds were able to stimulate the growth of several transformed cell lines other than by the TfR cycle by using haem or haemin (Ward *et al.*, 1984), soluble iron salts (Rudolph *et al.*, 1985), or chelators (Taetle *et al.*, 1985). Iron-binding proteins have been identified in the membrane of neoplastic and neoplasma-derived cell lines. The p97 molecule, a glycoprotein found in melanoma cells which has a high degree of homology with Tf could be an example of a

membrane-located iron binding protein that could participate in such a mechanism (Brown *et al.*, 1982).

The finding that FeNTA was able to allow proliferation of CCRF-CEM cells might also be explained by the possibility that these cells synthesise Tf, in line with the idea that iron from synthetic chelates is taken by the cells after exchange with cell associated Tf (Hemmapardh and Morgan, 1974). FeNTA has been shown to deliver iron into hepatoma cells, and it is known that FeNTA loads apoTf quite efficiently (Bates and Schlabach, 1973) and that contaminating exogenous Tf can affect any experiment studying iron uptake (Brock, 1989). Perhaps the growth stimulation of hepatoma cells seen with FeNTA which was mentioned above is due to this loading of apoTf, a protein which hepatocytes synthesise and secrete. In the present study, this suggestion is supported by the high proliferative background of CCRF-CEM cells cultured in the medium alone, suggesting that these cells are very efficient in taking advantage of the unavoidable iron contamination of the media and using it, perhaps by secreting their own Tf. This hypothesis supports the finding that blockade of TfR of these very same cells (CCRF-CEM) by the monoclonal antibody 42/6 inhibited cell growth and that the addition of ferric complexes or ferrous sulphate to cultures did not overcome the inhibitory effects of the antibody (Trowbridge and Lopez, 1982). However a further report by the same group provided evidence that the addition of soluble iron in the form of FeNTA results in partial reversal of inhibition of cell growth induced by 42/6 antibody in PHA-stimulated lymphocytes (Mendelsohn *et al.*, 1983).

To check this hypothesis Tf synthesis by these cells was examined.

The present study clearly demonstrated the synthesis of Tf by the CCRF-CEM cell line. The Tf produced may act as an autocrine promoter of cellular proliferation in a similar fashion to a number of other growth factors synthesised by malignant cells (Carney *et al.*, 1985). However one may wonder about the necessity of Tf synthesis as a requirement for proliferation of these cells, which had been cultured routinely in serum-containing medium for several months before performing the study. It could be that unlike unstimulated lymphocytes (see section 2.5.4), the Tf gene in these cells is constantly switched on as they are constantly dividing. The gene could be switched on as a preventive measure in case of shortages in the iron supply since withholding iron is a characteristic of host defence mechanisms against neoplasia (Weinberg, 1984; Letendre, 1985). The same thing applies to cells reaching high densities in cultures where availability of iron becomes a limiting factor. Synthesis of Tf would enable them to continue to grow under these conditions. Kitada and Hays (1985) have described two cloned murine malignant T-lymphoma cell lines which grew in serum-free medium without added Tf. These cells produced a Tf-like activity essential for cell proliferation. Another Tf-like growth factor produced by lymphoma cells has been reported by Morrone *et al* (1988). This factor supported autocrine growth of these cells, but inhibited normal T-lymphocyte proliferation, in effect serving to provide a substantial growth advantage for the malignant cells. In all the studies mentioned above, Tf synthesised by the cells has marked functional and biochemical similarities to native serum Tf.

If the amount of Tf synthesised by these cells was sufficient to

bind the large amount of FeNTA added to the culture medium and permit growth under those conditions, then it must also be sufficient to bind the trace amounts of endogenous contaminating iron present in the medium. In this case addition of apoTf should not make any difference. However addition of apoTf caused considerable increase in the rate of proliferation. It seems therefore that loading of iron from FeNTA is possible only for cells with an abundant endogenous Tf production sufficient to chelate most of the iron bound to NTA. Hence the existence of an alternative pathway for FeNTA acquisition by these cells, as well as that of the TfR pathway seems to be quite likely. Mouse leukaemic cells exhibit a Tf-independent iron transport system, which appears to be involved in the ability of these cells to take up chelate iron and use it to promote DNA synthesis and cell growth (Taetle *et al.*, 1985). This suggests that this uptake must occur through a regulatory transport system and not by mere diffusion of the chelator. The finding of a siderophore-like growth factor secreted by SV-40-transformed 3T3 mouse cells provides evidence for the existence of a specific low molecular weight transport system for iron and other trace metals (Fernandez-Pol, 1978). This factor was shown to induce DNA synthesis and proliferation of the cells.

The finding that these cells managed to grow at high levels of iron compared to their normal counterpart could suggest the hypothesis that unlike normal lymphocytes, these cells have the ability to synthesise increasing amounts of Ft in response to non-Tf bound iron. However this hypothesis could not be substantiated since CCRF-CEM cells were found to have extremely low intracellular Ft levels compared to their normal counterpart. The reason of this difference could lie in the differences in the degree of proliferation. It is well known that iron in these

proliferating cells is mainly needed for the functioning of the enzyme ribonucleotide reductase. Therefore, the amount of iron used by the cell will be proportional to the uptake of thymidine which directly measures DNA synthesis, and since the proliferative activity of CCRF-CEM cells was much higher than that of PHA-stimulated normal lymphocytes (~12X at optimum proliferation on a cell to cell basis; see sections 3.3.2 and 3.3.9), it is likely then that the former were able to use larger amounts of iron to sustain this high blastogenic activity. The intracellular low molecular weight iron pool which is supposed to be the precursor of incorporation into Ft (Jacobs, 1977) and which could mediate cytotoxicity is probably very limited. This could explain why these cells were not synthesising as much Ft as their normal counterpart and could also account for the resistance of these cells to the oxidative injury in the presence of high iron concentration unlike normal lymphocytes. It could also be that these cells synthesise mainly H-rich Ft which could not be detected by the assay used in this study. It has been shown that transformed cells synthesise H-rich Ft predominantly (Dörner *et al.*, 1983a). This type of Ft has been shown to uptake iron more readily than L-rich Ft at pH 7.0 (Levi *et al.*, 1989), probably because of the presence of a ferroxidase site on the H-chain.

As with normal proliferating T-lymphocytes, Lf was found not to have any effect on the growth of CCRF-CEM cells when added alone whether it was "iron-free" or iron-loaded. However, Hashizume *et al.*(1983) have reported that several human, but not mouse B and T-cell lines proliferated in serum-free medium supplemented with Lf. However in their study, B-cell lines generally grew better than T-cell lines, and

they found that growth of CCRF-CEM cells in Lf medium was only 20% over that found in serum-containing medium. This figure represents nearly the proliferative background of these cells cultured in the medium alone, found in the present study.

On the other hand, CCRF-CEM cells unlike their normal counterparts were found to be able to proliferate in the presence of FeNTA. This could also be explained by the possibility that these cells may synthesis abundant amounts of their own Tf (see below) or more likely could be due to a low-affinity iron-uptake system not involving Tf which might operate in these cells as has been reported previously (Basset *et al.*, 1986).

GENERAL DISCUSSION

The work reported in this thesis has attempted to define more closely the role of iron in the proliferation of lymphocytes *in vitro* with particular reference to the intracellular biochemical events occurring following its uptake. Few have looked at iron metabolism in lymphocytes, despite its importance in understanding how lymphocytes react to different degrees of extracellular iron availability. This study has demonstrated that the availability of Tf-bound iron in the extracellular medium and in particular the degree of Tf saturation has a critical effect on lymphocyte proliferation in response to mitogens. Iron donated in this form was used by the cell and permitted an increase in cellular metabolism as the largest proportion of iron donated to the cell in this form was found in a subcellular fraction probably consisting of enzymes and haem-containing proteins. However, when cells were cultured in the presence of high concentrations of iron (beyond the level that saturates all Tf present in the medium) proliferation was inhibited probably by affecting preferably the helper subsets, suggesting that some mechanism may exist for the uptake of the unbound iron into these cells, resulting in toxic consequences. Since cells cultured in the presence of 100% saturated Tf exhibit optimum transformation, this rules out the possibility that the inhibitory effect of iron seen at those high concentrations is a result of excess accumulation of iron taken up from rapidly endocytosed diferric Tf as previously suggested (Brock 1981). On the other hand, the effect of non-Tf bound iron in the form of FeNTA was clearly neutralized by the presence of apoLf in the medium.

It is well known that iron enhances the formation of toxic oxygen free radicals (Biemond *et al.*, 1988). The occurrence of damage due to oxygen free radicals depends on the balance between the amount of radicals which are formed and the amount of protection at the same location. The generation of these toxic oxygen intermediates, in particular hydroxyl radicals responsible for the peroxidation of membrane lipids might decrease in the presence of iron binding proteins because catalytically active coordination sites of the metal diminish. Plasma Tf has considerable reserves for coping with increasing amounts of incoming iron, but these may be exceeded in certain pathological conditions. Cultures containing the FeNTA complex in the present study perhaps provide a system comparable to the non-specific iron found in thalassaemic sera. In severe iron-overloaded haemochromatotic patients, Tf is completely saturated and as a result an abnormal serum iron fraction appears as low molecular weight iron, which has been reported to be present, loosely bound to a variety of serum protein such as albumin or non-specifically bound to Tf itself (reviewed by Hershko and Peto, 1987). Iron in the circulation in excess of that required to saturate available Tf is associated with increased susceptibility to infection. This is not only due to the fact that such iron complexes are more readily available to microbial-iron scavenging mechanisms than Tf-bound iron (Weinberg, 1978), but also to the possibility that this iron might interact with immune cells and affect their function. Because lymphocytes appear to lack the buffering effect of increased Ft synthesis against the excessive entrance of iron into the cell as discussed in section 3.4.2, they are particularly vulnerable to iron-dependent oxidative injury. As mentioned above the presence of non-Tf-bound iron, but not iron-saturated Tf,

tends to decrease the response of mouse and human lymphocytes to mitogens. Similarly a decreased response of lymphocytes to PHA and Con A was reported in thalassaemia patients with very high iron levels (Munn *et al.*, 1981; Dwyer *et al.*, 1987). Lymphocytes in liver, a tissue recognised for its high iron content, have been reported to respond poorly in the mixed lymphocyte reaction (Mukhopadhyay *et al.*, 1978). This observation raises the possibility that *in vivo* iron can also cause inhibition of lymphocyte responses under normal conditions. All this is in agreement with the findings of Kraut and Sagone (1981) who demonstrated that oxidant injury to lymphocytes impairs both membrane and cellular function such as their ability to form E-rosettes and impaired cap formation after binding of Con A as well as impaired transformation and cytotoxic capacity.

Iron loading can place a limit on the degree of activation of lymphocytes apparently by reduction of functional helper T cells. The $CD4^+$ subset plays a crucial role in the initial stages of development of immune responses. They are particularly important in regulating either directly or indirectly the functional activity of various other immune cells, including B lymphocytes, monocytes/macrophages, cytotoxic T lymphocytes, and natural killer cells. Therefore, the quantitative defect of these vitally important cells and the subsequent functional defect that this might cause in specific immune surveillance is of great physiological importance and may lead to immunosuppression. Thus, the decrease in the ratio of CD4/CD8 seen when high levels of iron were present in the culture medium suggests that this could be a handicap to the infected host as the decrease in proliferating cells, especially helper cells, could not help to combat the effect of the increased free iron available to

microorganisms. This is particularly relevant to iron overloaded patients in whom increased susceptibility to infection has been reported (idiopathic haemochromatosis or transfusional iron-overload thalassaemia) (Weinberg, 1978). As many as 12% of idiopathic haemochromatotic patients were reported to die with pneumonia, and severe bacterial infections are an important cause of morbidity and mortality in thalassaemia major (Hershko *et al.*, 1988). Cell mediated immunity appears to be particularly impaired in uraemic subjects who have received previous blood transfusions (Watson *et al.*, 1979). Allograft survival following renal transplantation is significantly prolonged in such subjects (Vincenti *et al.*, 1978; Watson *et al.*, 1979). The degree of improvement of kidney survival has been directly related to number of transfusions received (Opelz and Persijin, 1981), which illustrates the suppressed status of their immune system and supports de Sousa's view that this is due to an iron-related immunosuppression (de Sousa, 1983). However, other authors have reported that this is not just due to iron overload (Woodruff and van Rood, 1983).

The findings reported in this study are in line with many clinical investigations carried out on iron-overload related disease. Kapadia *et al* (1980) reported a decrease in number of T-helper cells and concomitant increase in B-cells associated with iron overload. Abnormally low numbers and functionally defective $CD4^+$ cells have been reported in thalassaemia intermedia (Guglielmo *et al.*, 1984), and in thalassaemia major (Grady *et al.*, 1985; Dwyer *et al.*, 1987; Pardalos *et al.*, 1987). In beta-thalassaemic children with very high transferrin saturation, the

ratio of CD4/CD8 was low compared to control, this decrease being due primarily to a reduction of $CD4^+$ cells and partly to an increase of the $CD8^+$ cells (Pardalos *et al.,* 1987). Similar findings as far as T-cell subpopulations are concerned have also been reported by others in beta-thalassemia patients (Neri *et al.,* 1984, Kessler *et al.,* 1983). In thalassaemia major the impaired production of immunoglobulins by B-cells was attributed to a defective T-helper cell population and not to the non-T-cell population (Nualart *et al.,* 1987). Iron overload has also been reported to influence the expansion of the $CD8^+$ cell population *in vivo*. In patients with thalassaemia major, whether splenectomized or not, an increase in the numbers of $CD8^+$ cells has been reported (Grady *et al.,* 1987). Other indirect evidence in favour of iron overload influencing expansion of the $CD8^+$ cell population *in vivo* comes from the observation of a negative correlation between the amount of DFO received and the numbers of $CD8^+$ cells present in thalassaemic patients (Dwyer *et al.,* 1987).

The reduction in the number of cells that are proliferating may be due to either cell death (cytotoxicity) as discussed above or to an impairment of proliferative capacity (suppression), or a combination of both. The catalytic effect of ionic iron upon lipid peroxidation could lead to alteration of the cell membrane and subsequently to cell death. It could also result in the generation of suppressor factors, which inhibit lymphocyte proliferation and may trigger the generation of antigen-non-specific suppressor cells (Fischer *et al.,* 1980). Conversely, the free oxygen radical species generated by iron might interact with suppressor cells to produce inhibitory factors. An example would be the interaction of these highly reactive species with arachidonic acid metabolism

resulting in prostaglandin synthesis which is known to modify T-cell functions and in particular to activate suppressor mechanisms (Hemler *et al.,* 1979; Dinarello *et al.,* 1983). This could explain the slight increase in the proportion of the $CD8^+$ subset when cells where exposed to non-Tf bound iron in the form of FeNTA. All this points to specific responses of T-cell subsets to changes in the extracellular iron concentration. However the mechanism(s) whereby iron seems to have a selective influence on T-populations remain unclear.

In inflammatory diseases local accumulation of iron occurs and may cause damaging free radical reactions (Blake *et al.,* 1983) which may similarly affect the immune system. Indeed uptake of ionic iron as the chelate FeNTA by rabbit synovial fibroblasts was accompanied by increased production of latent collagenase and prostaglandin G_2 (Okazaki *et al.,* 1981). Addition of DFO prevents the whole process. On the other hand, the combination of the decrease in T helper/inducer cells and increase in the T suppressor/cytotoxic cells may be a protective mechanism as part of the normal accelerated acute phase response may be avoided. However, prolonged depression of T cell immune response could be a handicap to the host against microbial invaders and may also be a major factor in the development of malignancies as discussed above.

Lf can prevent potentially toxic effects of iron as a result of its ability to dampen tissue-damaging free radical reactions. When neutrophils phagocytose bacteria, particles, or immune complexes, they show a rapid burst of oxygen uptake associated with the production of oxygen derived species such as superoxide $O_2^{-\cdot}$ and hydrogen peroxide H_2O_2 (Babior, 1978). In the presence of trace amounts of iron, $O_2^{-\cdot}$ and

H_2O_2 react together to form the hydroxyl radical $OH^{\cdot}$ which participates in the bacterial killing mechanism but can also attack and damage almost all biological molecules and induce lipid peroxidation (reviewed by Halliwell, 1989; Hershko, 1989). In contrast to Tf, Lf can bind iron tightly at acid pH values and thus can function well in tissue sites in which the pH has been lowered by microbial and leucocytic metabolism. When released into the phagocytic vacuole, Lf can be expected to sequester any free iron and thus limit the damage caused to surrounding tissues. It might therefore be possible that secretion of Lf from neutrophils in large amounts during phagocytosis might be a mechanism by which surrounding tissues are protected against oxidative injury. This view is consistent with the report that iron-free or partially saturated Lf inhibits lipid peroxidation (Gutteridge *et al.*, 1981; Sibille *et al.*, 1987). This could be particularly important during inflammation, since synovial fluid from patients with inflammatory disease contains free iron and large numbers of phagocytic cells infiltrating at the site of inflammation (Wong *et al.*, 1981; Gutteridge *et al.*, 1981), at which radicals have been implicated in the pathogenesis of inflammation (Sacks *et al.*, 1978). Lf is found extracellularly at sites of inflammation and its levels have been reported to correlate directly with the degree of inflammation of rheumatoid joints (Bennett and Skosey, 1977). There is little data about iron saturation of Lf in neutrophil granules, but it is usually considered to be iron free (van Snick *et al.*, 1974; Bullen, 1981).

During an inflammatory response there must be a balance between inducing factors such as endotoxins and other infective agents or

complement breakdown products, which provide a positive stimulus to mononuclear cells, and negative regulation factors. Lf could be one of the latter. A possible extracellular role could be attributed to Lf when neutrophils are activated, as it could provide a negative feedback control of lymphocyte growth possibly by depriving them of iron in the medium, which is in keeping with other evidence that Lf inhibits T-cell function (Bagby *et al.,* 1981) and antibody production (Duncan and McArthur, 1981). Lf could be particularly important in neutralizing the harmful effect of local iron excess in inflammation as discussed above. On the other hand, iron overloaded patients could be facing a double problem; iron availability to invading microorganisms from one side, and the inefficiency of the protective effect of Lf which under these conditions became saturated with iron. In these cases, Lf might obtain iron from the iron rich milieu, which contains fully saturated Tf and low molecular weigh iron compounds. In addition pinocytosed Tf which in contrast to Lf might also release bound iron readily as the pH drops to levels easily achieved in the phagocytic vacuole (Lestas, 1976) could also contribute to loading Lf with iron within the phagocytic vacuole of neutrophils.

Many areas of research in this field still need to be examined, including whether Lf is internalized and cycled through cells, and if so whether it cycles with Tf or by a different route. It would also be of interest to examine the effect of Lf on iron uptake by cells. The recent finding of Furmanski *et al* (1989) of the existence of several isoLfs exerting different functions might open an exciting chapter in understanding the various and complicated functions of this protein and could lead the way to many interesting investigations.

The work presented here has tried to establish the effect of different forms and levels of iron on mouse and human cells *in vitro*. It would be of interest to extend this work to an *in vivo* system using an experimental animal model which could probably provide a more complete picture of the effect of different levels of iron on lymphocytes and the immune system as a whole, and also a more adequate explanation of these effects.

The present study showed that the lymph node macrophages, which increased in number upon immunological activation have the capacity to synthesise Tf. This Tf could augment the iron supply of rapidly proliferating lymphocytes. For locally-synthesised Tf to be of benefit in immunological activation it would probably need to contain iron. Although hepatic Tf is generally thought to be synthesised and released in the iron-free form (Hemmapardh and Morgan, 1974) it has been hypothesized that iron released from macrophages is transported by Tf of endogenous origin (Haurani and Ballas, 1984), and it is therefore of particular interest that iron released by macrophages is increased by treatment *in vivo* with *Corynebacterium parvum* (Alvarez-Hernandez *et al.*, 1986) or *in vitro* with γ-IFN (Taetle and Honeysett, 1988). Therefore in the future it would be of prime importance to investigate whether Tf synthesised by macrophages contains iron and to establish whether Tf synthesised by these cells can enhance lymphocyte proliferation which would open an area of possible immunoregulatory activity. This would in turn fundamentally affect wider issues such as the controversial question of whether iron deficiency affects immune function, and whether iron depletion is beneficial to immune function and resistance to infection. Further studies are also necessary in order to

examine the effect of various agent that would affect Tf synthesis by these cells. Conversely, CCRF-CEM, a T-leukaemic cell line has been found to produce Tf. These findings support the hypothesis (Fernandez-Pol, 1983) that growth modulating factors like siderophore-like growth factors and Tf-like activity synthesised and secreted by some transformed cells may enable tumour cells to take advantage of local conditions, and decrease their dependence on serum factors (Tf in this case), and compete with normal cells for specific trace metal ions. Moreover the findings of this study suggests that these cells, unlike their normal counterparts exhibit Tf-independent iron transport which appears to be involved in the ability of these cells to take up iron in a variety of forms and to use it efficiently for cell growth. Recently, Sturrock, *et al.*, 1990) have demonstrated that uptake of non-Tf bound iron by HeLa cells from various chelates was Tf-independent. This iron was used by the cells as most of it was recovered in haem and Ft. These findings lend some indirect support to the frequent reports that patients with iron overload-related diseases are vulnerable to development of neoplasia (reviewed by Weinberg, 1985), as non Tf-bound iron probably exists in these patients.

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